Casein Proteins as a Vehicle to Deliver Vitamin D$_3$: Fortification of Dairy Products with vitamin D$_3$ and Bioavailability of Vitamin D$_3$ from Fortified Mozzarella Cheese Baked with Pizza

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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ABSTRACT

Current vitamin D intakes in Canada are inadequate. The extension of vitamin D fortification to additional foods may be an effective and appropriate strategy for increasing vitamin D intakes in the general population. Cheese is potentially an ideal candidate for vitamin D fortification. We introduce the potential use of casein proteins as a vehicle for vitamin D₃ fortification in industrially made cheeses where we found that over 90% of vitamin D₃ added to milk was retained in both Cheddar and Mozzarella cheeses. Use of casein proteins for vitamin D₃ fortification did not fully prevent vitamin D₃ loss into whey. However the loss was minimized to approximately 8%. We then show that vitamin D₃ is bioavailable from fortified Mozzarella cheese baked with pizza suggesting that the high temperature baking process does not significantly breakdown vitamin D₃. Our findings could have important implications in increasing fortified food options for Canadians.
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Contributions

The vitamin D concentrated casein premix was made in-house by me at the Food Science Research lab, Ryerson University, Toronto, Ontario.

The fortified cheeses were made by Agropur Cooperative at the Agropur Research and Development Centre (ARDC) in Saint-Hyacinthe, Quebec. I was present at the ARDC during cheese production and performed the vitamin D₃ fortification phase. I also helped the Agropur personnel in manufacturing the fortified cheeses. I based the fortification procedure on preliminary lab-scale cheese model system testing the suitability of the new fortification protocol, as well as followed the method of Wagner et al [1] for industrial scale cheese production. I made the required calculations and protocol revisions under the guidance and direction of Dr. Reinhold Vieth (University of Toronto) and Dr. Derick Rousseau (Ryerson University).

I performed all the food science experiments under the direction of Dr. Vieth and Dr. Rousseau, performed the statistical analysis and wrote the manuscript presented here, which will soon be submitted to the Journal of Agriculture and Food Chemistry.

The bioavailability trial was the second major part of my thesis. I participated in recruiting the subjects, obtaining written informed consent, taking blood and urine samples at the appropriate visits, helping with pizza baking at George Brown College, and collecting data. I also analysed all the serum samples collected from the subjects for 25(OH)D concentration using the DiaSorin Liaison method. The remaining blood and urine biochemistries were done at Mount Sinai Hospital’s clinical chemistry laboratory. I analysed the results of the pizza bioavailability study and wrote the manuscript presented here, which will soon be submitted to the Journal of Nutrition. I already presented the bioavailability data as a poster at Experimental Biology 2012 conference (San Diego, California).
Acknowledgements

We would like to thank the Dairy Farmers of Canada for supporting this research project.

We sincerely thank Agropur Cooperative ® for making and supplying the fortified cheeses. In particular we would like to express our gratitude to Pierre Morin, an Agent at Agropur Research and Development, and Michel Pouliot, the director of Agropur Research and Development, for helping us in this project.

We sincerely thank George Brown College, Centre for Hospitality and Culinary Arts for their collaboration in this research project and supplying pizza meals for the bioavailability study. A special thanks to Winnie Chiu, director of Food Innovation and Research, Moira Cockburn, Applied Research food scientist, and Candace Rambert, culinary technician, and George Brown College culinary students for their excellent work during the bioavailability trial.

I would like to express my sincere gratitude to Dr. Reinhold Vieth and Dr. Derick Rousseau for their invaluable guidance and mentorship. I would also like to extend my thanks and appreciation to Dr. David Cole and Dr. Tom Wolever, who were very helpful and insightful throughout my work, and my labmates Samantha Kimball, Dennis Wagner, and Nadeen Taha for their valuable insight, support, and encouragement throughout my research studies.

We thank Dr. Azar Azad, and Michelle Rodrigues of Mount Sinai Services, as well as their staff, for their excellent organization efforts and laboratory work in analyzing the blood and urine biochemistry tests.

Lastly, we would like to thank the 96 people who participated in the bioavailability trial for their time and commitment.
Chapter 1 : Introduction
1.0 Introduction

Vitamin D deficiency and insufficiency are leading health concerns in North American populations, as vitamin D malnutrition still remains an international health care priority. The media and the public are becoming more aware of the beneficial roles of vitamin D. Physicians have been ordering more and more blood vitamin D tests, suggesting that vitamin D deficiency is becoming a public health concern. Chronic vitamin D insufficiency prevents the proper mineralization of bone, leading to rickets in children and osteomalacia or osteoporosis in adults. Low serum vitamin D concentrations have been associated with several chronic and infectious diseases, including cancers [2-8], multiple sclerosis, diabetes [9-14], cardiovascular disease [15-17], rheumatoid arthritis [18], and microbial infections [19-21]. During the last ten years, there has been increasing interest in the potential roles of vitamin D in human health. With a growing body of evidence, the institute of medicine (IOM) has recently conducted a thorough review of all available evidence to revise the dietary guidelines for vitamin D and calcium [22]. The newly established DRIs for vitamin D pose a challenge for Canadians given the insufficient amounts of vitamin D present in our foods and minimal sun exposure in winter months, particularly in regions with higher latitude. It is clear that current intakes of vitamin D in North American populations are not adequate to meet the new recommendations for vitamin D [23-26]. Policy makers at Health Canada advise Canadians to avoid skin exposure to sunlight, hence the only source of vitamin D for the majority of the Canadians is dietary sources either through food fortification or consumption of supplements. With the current revised DRIs for vitamin D, reliance on fortified foods and dietary supplements is necessary to meet the new vitamin D requirements. Advising the population to take a vitamin D supplement is not an effective strategy to make a significant contribution to public health because of the associated costs, poor adherence among healthy people, and lack of awareness among the most vulnerable population groups. For a more effective intervention across a broader population, the food system needs to be changed in Canada to increase fortified foods options and allow much higher levels of vitamin D fortification [27, 28]. My thesis research is timely given with the recently revised dietary recommendations for vitamin D. We chose to fortify Cheddar and Mozzarella cheeses with vitamin D₃ at 2 different fortification amounts; high dose fortification level delivering 28,000 IU/serving and low dose fortification level delivering 200 IU/serving. In Canada, industrial milk
destined for cheese manufacture is not fortified with vitamin D. Cheese is an ideal candidate for vitamin D fortification for several reasons including its wide consumption in North American populations, high nutritional value, consumption by many lactose-intolerant individuals, and longer shelf life than milk.

Previously, our research group demonstrated that industrially made Cheddar and low fat cheeses are suitable dairy products for vitamin D₃ fortification [1], and raise vitamin D status as effectively as a vitamin D supplement [29]. These previous findings demonstrate that cheese is a good vehicle for fortification and delivery of vitamin D. However, from a dairy industry perspective, the partial loss of vitamin D in whey, particularly in the low-fat cheese was undesirable, making the whey byproduct unmarketable for producers. Therefore, my thesis project aimed to develop and apply a fortification protocol to preferentially bind vitamin D₃ to milk caseins as a delivery system for vitamin D₃ fortification to optimally deliver all of the casein-bound vitamin D₃ into cheese and reduce the loss in whey. I assessed the vitamin D₃ retention of the fortification protocol in a cheese model system and in industrial-scale Cheddar and Mozzarella cheeses. The results of the “food science component” of my thesis are described in Chapter 4, and are presented as a manuscript I wrote entitled “Vitamin D₃ fortification of industrially-made Cheddar and Mozzarella cheeses using caseins as a delivery system.”

The next phase of my work comprised of the “clinical nutrition component” of my thesis. The purpose was to determine whether vitamin D₃ is bioavailable from fortified Mozzarella cheese when baked with pizza in an oven at high temperature. We conducted a randomized controlled trial (RCT) of 96 healthy Canadian adults who consumed vitamin D₃ fortified Mozzarella cheese baked with pizza, randomly allocated to either receive 28,000 IU vitamin D₃/wk or 200 IU vitamin D₃/wk for 8 weeks during the winter. Bioavailability was assessed by comparing serum 25(OH)D responses between baseline and by the end of the study protocol. The RCT investigating the bioavailability of vitamin D₃ from Mozzarella cheese is described in Chapter 5, and are presented as a manuscript I wrote entitled “The bioavailability of vitamin D₃ fortified Mozzarella cheese under high temperature pizza baking process.”
Overall, we hoped to validate the suitability of vitamin D₃ fortification of cheese using milk caseins as a delivery system for vitamin D₃ fortification, and particularly validate a fortification level that aligns with the new dietary guidelines for vitamin D.
Chapter 2 : Literature Review
2.0 Vitamin D and Health

2.1 Historical Perspective

Rickets, synonymous with vitamin D deficiency, was scientifically first described in 1650 by Glisson, DeBoot, and Whistler as a bone disease identified by deformities of the skeleton [30]. It was first documented in Northern Europe during the mid-17th century of industrial revolution where people lived in an environment that was devoid of direct exposure to sunlight.

The first observation into the potential mechanism and the role of sunlight in curing this bone-deforming disease was documented by Sniadecki in 1822 [31]. He reported that those children living in the urban areas of Warsaw, Poland, had a high incidence of rickets, while children living on the farms surrounding Warsaw did not have the disease. He accurately concluded that it was the lack of sunlight in the urban areas of Warsaw that was the likely cause of rickets. Later in 1890, in an epidemiological study investigating factors that might associate with rickets, Palm further confirmed the cause of rickets due to insufficient exposure to sunlight [32].

Using a nutritional approach, Bretonneau in the mid-1800s effectively treated a 15-month-old child with acute rickets by administrating cod liver oil [30]. Later, a student of Bretonneau, Trousseau, used liver oils from a variety of fish and marine animals for the treatment of rickets and osteomalacia [33]. These clinical observations led to the concept that rickets was caused by a nutritional deficiency. Disregarding these important clinical observations, rickets had become epidemic in industrialized cities of northern Europe and the northeastern region of the United States by the turn of the 20th century. It was not until 1918 that the scientific community began to consider rickets as a nutritional deficiency disease when Mellanby fed rachitic dogs cod liver oil which successfully cured their rickets [34]. He concluded that cod liver oil contained an antirachitic factor that was fat-soluble, which was thought to be vitamin A at the time. However, McCollum et al. clearly demonstrated that when cod liver oil was exposed to heat and oxygen, it destroyed the vitamin A activity without affecting its antirachitic activity [35]. After these findings, McCollum established a new name for the fat soluble antirachitic factor in cod liver oil which was subsequently named vitamin D. About the same time, working under the findings of Mellanby’s observations, Huldshinsky and Hess et al [36] independently showed that rachitic
children could be cured with exposure to artificial radiation from a mercury vapor arc lamp or sunlight.

Following these findings, Goldblatt and Soames [37, 38], Hess and Weinstock [39], and Steenbock and Black [40] exposed a variety of foods, such as wheat, lettuce, olive, linseed oils, and rat chow and other substances, such as human and rat plasma, to ultraviolet (UV) radiation. These findings ultimately led to the addition of synthetic vitamin D directly to milk. This simple concept led to the elimination of rickets as a significant health problem in the United States and other countries that used this practice. Thus nearly one century after Sniadecki, it was finally demonstrated that exposure to sunlight or ingesting ultraviolet irradiated foods could prevent and cure rickets.

2.2 Photobiology of Vitamin D

There are 2 forms of vitamin D. Early discoveries relating to the antirachitic activity that could be produced by exposure of skin or foods to UV radiation led scientist to isolate and identify the precursor of vitamin D. The first one identified was Ergosterol from yeast, which is the provitamin D for vitamin D\textsubscript{2}. Ergosterol is a major sterol found in fungal and plant kingdoms. After UVB irradiation, the Ergosterol in mushrooms and yeast is converted to vitamin D\textsubscript{2}. Originally, it was thought that vitamin D\textsubscript{2} was the same form of vitamin D produced in the skin after sunlight exposure. However, vitamin D\textsubscript{2} was found to be less effective in curing rickets compared to vitamin D in pig skin. This finding led Windaus and Bock to isolate 7-dehydrocholesterol (7-DHC) from skin, which is the provitamin D for vitamin D\textsubscript{3}[41]. Vitamin D\textsubscript{3} is also found in oily fish and oils from the liver of cod and polar bear. The only difference between the two forms of vitamin D is that Vitamin D\textsubscript{2} has an extra double bond between carbons 22 and 23, and a methyl group on carbon 24 (Figure 2.1).
Figure 2-1 The chemical structures of vitamin D₂ and vitamin D₃.

During exposure to sunlight, 7-DHC naturally found in the epidermis and dermis layers of the skin, absorb UVB radiation with wavelengths between 290 and 315 nm causing a bond cleavage between carbon 9 and carbon 10 to form a 9,10-secosterol called previtamin D₃. Once previtamin D₃ is synthesized in the skin, it can undergo either a photoconversion to lumisterol, tachysterol, and 7-DHC or heat isomerized to vitamin D₃. The heat isomerization of previtamin D₃ to vitamin D₃ is the last step in the synthesis of vitamin D₃ in human skin. The reaction rate of this isomerization step is temperature dependent and is enhanced by raising the temperature. After vitamin D₃ is formed, it is transported into the dermal capillary bed beneath the dermoepidermal junction where it is bound to vitamin D binding protein (DBP) and enters the circulation [42]. An adult in a bathing suit exposed to one erythemal dose of ultraviolet radiation (a slight pinkness in skin that occurs 24 h after UV radiation) is reported to be equivalent to ingesting between 10,000- 25,000 IU of vitamin D [30]. This is likely the reason why vitamin D is virtually absent from our food supply, because from an evolutionary perspective humans have depended largely on sun for their vitamin D requirements. However, a variety of endogenous and environmental factors could potentially limit the skin’s production of vitamin D₃. An increase in skin pigmentation (melanin) and use of topical sunscreen will absorb solar UVB photons reaching the skin, thereby significantly reducing the production of vitamin D₃ in skin by as much as 99%. Aging reduces the production of vitamin D₃ due to decreased concentration of 7-DHC in the skin. Change in latitude, season of the year, or time of the day can alter the zenith angle of the sun which also affects the amount of UVB photons reaching the skin. Skin synthesis of vitamin
D is absent during most of the winter months in populations living at latitudes outside the tropics (>30°N and >30°S). In these populations, reliance on dietary sources of vitamin D becomes a major factor in determining their vitamin D status during limited skin synthesis of vitamin D.

2.3 Vitamin D Absorption and Transport into Circulation

Vitamin D\textsubscript{2} and vitamin D\textsubscript{3} from dietary sources are emulsified with bile salts and solubilized within micelles in the duodenum which are then passively absorbed in the jejunum along with other lipids [43]. After absorption, vitamin D is incorporated into chylomicrons which are then transported into the venous circulation via the lymph system. Once in the circulation, vitamin D is bound to the vitamin D-binding protein (DBP) which transports it to various vitamin D target sites.

DBP is the major serum transport for vitamin D and its metabolites in the circulation. Like other serum proteins, DBP is made primarily in the liver where 10mg/kg/day of DBP is estimated to be produced in humans [44]. DBP associates with a number of biological molecules including lipoproteins and albumin but these associations are weak and nonspecific. Vitamin D sterols form a specific, high capacity, high affinity association with DBP [45]. In the circulation, DBP is present in large excess amounts relative to its vitamin D ligands, with only 5% of DBP molecules being occupied by vitamin D sterol ligand [46]. All forms of vitamin D sterols (D represents D\textsubscript{2} or D\textsubscript{3}) are bound to DBP by a single binding site, but with variable affinity [47]. DBP shows the highest affinity for 25(OH)D and 24,25(OH)\textsubscript{2}D, followed by 1,25(OH)\textsubscript{2}D and the parent vitamin D [48-51]. Vitamin D binding results in the rotation of domain I structures of DBP leading to chemical and thermal stability of DBP [45, 52]. The total binding capacity of DBP has been reported to be approximately 4700 nmol/L for vitamin D metabolites [48] and this specific, high affinity association with DBP is thought to facilitate their dispersal throughout the body, and their delivery to sites of metabolism and biological actions.

2.4 Metabolism of Vitamin D

A schematic representation of the vitamin D metabolic pathway is illustrated in Figure 2.2. Vitamin D either made in the skin or ingested in the diet is transported to the liver via DBP. In the liver, vitamin D is enzymatically hydroxylated on carbon 25 to form 25-hydroxyvitamin D.
[25(OH)D] [53, 54], which is the major circulating metabolite of vitamin D. The enzyme for this step has not yet been identified. However, a mitochondrial CYP27A1 has been shown to be capable of 25-hydroxylating vitamin D when the substrate is present in high concentrations [55]. Through a single pass in the liver, approximately 75% of circulating vitamin D is 25-hydroxylated and this process is not significantly regulated [56]. Increasing intake of vitamin D results in higher blood levels of 25(OH)D, although not in a linear manner [57, 58]. 25(OH)D is then transported to the kidney where it is enzymatically hydroxylated by CYP27B1 on carbon 1 to form 1,25-dihydroxyvitamin D [1,25(OH)2D], the active hormonal metabolite of vitamin D. 1,25(OH)2D is classified as a hormone because its production is at a distant site from its target action sites, and its production is highly regulated by a feedback mechanism of serum calcium and phosphorous levels through parathyroid hormone to prevent hypercalcemia and hyperphosphatemia [59]. Regulation of the vitamin D endocrine system occurs through the stringent control of renal CYP27B1 activity, maintaining 1,25(OH)2D in strict homeostatic range despite varying amounts of 25(OH)D substrate. The increase in PTH in response to low serum calcium levels stimulates the production of the CYP27B1 enzyme to increase the synthesis of 1,25(OH)2D [60]. Furthermore, low serum calcium or phosphorus may activate CYP27B1, independent of PTH [61]. To prevent excess synthesis, 1,25(OH)2D induces its own destruction by stimulating the 24-hydroxylase enzyme(CYP24A1), which catabolizes both 25(OH)D and 1,25(OH)2D into biologically inactive, water-soluble 24,25-dihydroxyvitamin D [24,25(OH)2D] and 1α 24,25(OH)2D, respectively [62, 63]. These products are excreted through the bile into the feces [62]. The 24-hydroxylase enzyme is expressed in all vitamin D target tissues with the highest concentrations found in the kidney and is induced in response to 1,25(OH)2D interacting with the intracellular vitamin D receptor (VDR). Hence, circulating levels of 1,25(OH)2D are highly regulated and involve reciprocal adjustments to the rates of synthesis and catabolism.
Figure 2-2  Schematic representation of the synthesis and metabolism of vitamin D. During exposure to sunlight (UVB), 7-dehydrocholesterol in the skin is converted to previtamin D$_3$ (preD$_3$) which is immediately converted to vitamin D$_3$ by a heat-dependent process. Dietary sources of vitamin D are absorbed from the intestine and are transported into the circulation. Vitamin D in the circulation is bound to the vitamin D-binding protein (DBP) which transports it to the liver where vitamin D is converted by the vitamin D-25-hydroxylase (25-OHase) to 25(OH)D. This is the major circulating form of vitamin D that is used by clinicians to measure vitamin D status. 25(OH)D is biologically inactive and must be converted in the kidneys by the 25-hydroxyvitamin D-1α-hydroxylase (1-OHase) to 1,25(OH)$_2$D, the biologically active form. 1,25(OH)$_2$D is involved in “classical” actions such as regulating calcium, phosphorous, and bone metabolism; as well it is involved in “non-classical” actions including cell growth regulation, immunomodulatory effects, and innate immunity. 1,25(OH)$_2$D feedback regulates its own synthesis and decreases the synthesis and secretion of PTH in the parathyroid glands. 1,25(OH)$_2$D increases the expression of 24-hydroxylase (24-OHase) to catabolize 1,25(OH)$_2$D and 25(OH)D to the water soluble biologically inactive 24,25(OH)$_2$D and 1α 24,25(OH)$_2$D which are excreted in the bile.
2.5 Physiologic Actions of Vitamin D

The classical function of vitamin D hormone is to regulate serum calcium and phosphorous levels required for skeletal mineralization to optimize bone health [64, 65]. The hormone mainly acts on the intestine, bone, and kidney to sustain metabolic and physiologic functions for bone health. The biological effects of 1,25(OH)$_2$D are induced by its interaction with VDR. Once the hormone enters the cell either by diffusion, or facilitated entry via megalin, or local synthesis, it binds VDR in the cytoplasm of the cell with high affinity. The VDR complex then binds the retinoic acid receptor (RXR) to form a heterodimer [66, 67]. The VDR-RXR heterodimer can then bind to vitamin D response elements (VDREs) in target genes to regulate their expression [68]. It is well established that VDR is expressed widely in the classic vitamin D target organs including intestine, bone, kidney, and the parathyroid glands [69].

1,25(OH)$_2$D acts upon the intestine to stimulate the active transport of calcium and phosphorus from the intestinal lumen to the serum [70-76]. In the bone, together with PTH it activates bone calcium mobilization [77, 78]. In addition, 1,25(OH)$_2$D and parathyroid hormone act upon the kidney to stimulate the active reabsorption of calcium from the urine to blood. Its role in phosphorous absorption is often stated where low serum phosphorus levels have been reported to trigger an increase in the activity of the 25(OH)D-1-hydroxylase enzyme but the mechanism remains unknown [79-81]. Once serum calcium levels have returned to normal, PTH secretion is no longer stimulated and, thus the production of 1,25(OH)$_2$D is no longer activated. Although the primary driving force of the vitamin D system is to regulate serum calcium and phosphorus for proper mineralization of the skeleton, the effects of vitamin D are not limited to skeletal health. It is well established that VDR is expressed widely in the classic vitamin D target organs (intestine, bone, kidney, and parathyroid glands) [69]. In addition to these classic target organs, the VDR and its ligands have been found in cells and tissues not playing a role in calcium and phosphorous homeostasis. In particular, the VDR is found in the islet cells of the pancreas, in activated cells of the immune system, in the macrophage, in epithelial cells of the intima of blood vessels, in cells of the stomach, in keratinocytes of skin, in epithelial cells of the colon, and in cells of the placenta [82-86]. In addition, the 1α-hydroxylase (CYP27B1) gene has been reported to be expressed in many extra-renal tissues, allowing them to locally synthesize 1,25(OH)$_2$D from 25(OH)D [87].
Insufficient levels of vitamin D have been implicated in the risk of overall mortality [88], cancer [89], diabetes [89], musculoskeletal disorders [89], physical performance [90], hypertension [89], cardiovascular diseases [89], and autoimmune diseases [89]. It has been reported that the local synthesis of 1,25(OH)₂D in macrophages results in an increase in cellular immunity by stimulating the production of an anti-microbial peptide capable of killing bacteria, particularly *Mycobacterium tuberculosis* [20]. The immunoregulatory effects of vitamin D may also play a role in cancer biology. Moreno et al demonstrated the ability of vitamin D to exhibit anti-inflammatory effects on cancer cells by down-regulating the pro-inflammatory pathways which may contribute to cancer inhibition [91]. The investigation into extra-skeletal health outcomes of vitamin D are still an active area of research as no consensus exits concerning the threshold for vitamin D status necessary to guarantee optimal health.

### 2.6 Dietary Guidelines for Adequacy and Current Intakes

The Institute of Medicine (IOM) in 2011 was requested to conduct a review on the growing body of scientific evidence about the roles of vitamin D and revise Dietary Reference Intakes (DRIs) for vitamin D [22]. The newly established dietary requirements for vitamin D mark the first DRI review since the completion of the 1997 report [92]. Previously, the dietary recommendation for vitamin D had been reported as “Adequate intake” (AI—essentially a preliminary approximation) which used a different approach to estimate vitamin D requirements. The newly established DRIs now incorporate a statistical distribution for set reference values that specify the Estimated Average Requirement (EAR), the Recommended Dietary Allowance (RDA), and a Tolerable Upper Intake Level (UL) of vitamin D for all life stage groups except infants up to 12 months of age for which an AI was specified [22]. These reference values specify nutrient requirements for normal, healthy populations living in North America with an assumption of minimal sun exposure. The EARs, RDAs, and AIs for vitamin D are shown in Table 2.1. The DRIs for vitamin D are based on measures of serum 25(OH)D levels which is a well-established biomarker for total vitamin D exposure from food, supplements and skin synthesis. When establishing these reference values, the IOM recognized only the skeletal health outcomes as relevant to dietary advice and health policy. These reference levels for 25(OH)D are as follows: concentrations <30 nmol/L are associated with the risk of deficiency, level of 40 nmol/L are consistent with the EAR of 400 IU/d, and of 50 nmol/L are consistent with the RDA of 600-800
IU/d for those aged ≥1 y. However, controversy over optimal serum 25(OH)D levels for health maintenance beyond skeletal health outcomes still remain a subject of debate. Previously, serum 25(OH)D levels > 75nmol/L were associated with multiple health outcomes, not only bone health [25, 93-99]. The US Endocrine Society states that serum 25(OH)D concentrations ranging from 75-110 nmol/L have been implicated in additional health benefits in reducing the risk of common cancer, autoimmune diseases, type-2 diabetes, cardiovascular disease, and infectious diseases [100].

Reliance on dietary sources of vitamin D is greatest in the winter at latitudes outside the tropics (>30°N and >30°S), and among people with restricted skin sunshine exposure [101, 102]. Living above latitude 35°N, most Canadians are not able to synthesize cutaneous vitamin D from sunlight exposure for 4-5 months of the year [89]. Looking at the dietary vitamin D intakes among Canadians (between ages 1 and >70) in the 2004 Canadian Community Health Survey Cycle 2.2 (CCHS 2.2); Canadians reported ingesting, on average, 200-300 IU vitamin D/d from foods alone [103, 104]. These reported average intakes for Canadians are 2-3 times less than the current RDA of 600 to 800 IU/d. These data should raise serious public health concerns for dietary intakes of vitamin D for Canadians. This is not surprising given there are no meaningful natural source of vitamin D in the kind of foods Canadians are consuming. The CCHS data clearly show that none of the subpopulations categorized under any age, sex, and ethnicity groups meet the current RDA for vitamin D. Males 9-18 years had the highest intakes (292 ± 4 IU/d) and females 51-70 years had the lowest intakes (204 ± 12 IU/d). Males in all age groups under 70 years had significantly higher intakes than females in the corresponding age groups.
Table 2-1 Vitamin D Dietary Reference Intakes (DRIs) for Adequacy (amount/day)

<table>
<thead>
<tr>
<th>Life Stage Group</th>
<th>AI</th>
<th>EAR</th>
<th>RDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 6 mo</td>
<td>400 IU (10 μg)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6 to 12 mo</td>
<td>400 IU (10 μg)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–3 y</td>
<td>—</td>
<td>400 IU (10 μg)</td>
<td>600 IU (15 μg)</td>
</tr>
<tr>
<td>4–8 y</td>
<td>—</td>
<td>400 IU (10 μg)</td>
<td>600 IU (15 μg)</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
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AI= Adequate Intake; EAR= Estimated Average Intake; RDA= Recommended Dietary Allowance. Table was adopted from reference [22].

According to the CCHS, milk products in Canada which are fortified with vitamin D at approximately 400 IU/L, contributed 116 IU vitamin D/d for all age/gender groups. For children 1-8 years, milk products contributed 75% of dietary vitamin D intake. The contribution declined by age over 9 years. Examining differences in vitamin D intakes by ethnicity, data from the CCHS showed significant difference between Caucasian and non-Caucasian groups only for males 9-18 years, although this may be that the non-Caucasian group was very diverse and supplement use was not reported. However, a study done by Gozdzik et al reported differences in total dietary intakes from foods and supplements between ethnic communities in Toronto, where
European, South Asian, and East Asian subjects had intakes of 231, 164, and 133 IU/d, respectively [105]. Similarly, it has been reported that aboriginal populations in Canada who have drifted away from their traditional diets to more western diets, and darker skinned immigrants of Asian ancestry consume significantly lower vitamin D from milk and dietary supplements [105, 106]. These findings demonstrate a clear and compelling reason to increase dietary intakes of vitamin D in Canada.

### 2.7 Vitamin D Inadequacy in Canada

The basis for the newly established RDA for vitamin D was determined as the daily amount of dietary intake that assures 97.5% of the general population will attain a serum 25(OH)D concentration ≥ 50 nmol/L in order to sustain bone density, calcium absorption, and minimize risk of rickets in children and osteomalacia in adults. The reference levels for serum 25(OH)D are based solely on bone health, disregarding further health outcomes because serum 25(OH)D levels beyond 50 nmol/L were reported to show no further health benefit. Before the release of the IOM’s 2011 report, there was a general consensus for serum 25(OH)D levels ≥ 75 nmol/L for multiple health outcomes, not only bone health [25, 99]. Previous efforts assessing optimal serum 25(OH)D concentration was defined as the concentration that maximally suppressed serum PTH and promoted maximum calcium absorption [99]. Bischoff et al. presented cross-sectional data, sampled from the National Health and Nutritional Examination Survey (NHANES), where there was a substantial benefit to bone mineral density (BMD) beyond 50 nmol/L and reaching a plateau at a concentration of 90-100 nmol/L in an older Caucasian population. Shifting the population to a threshold level greater than 75 nmol/L, would result in an increase in BMD by up to 4-5% in younger and older adults, an increase in lower extremity function by up to 4-6% among older adults, reduce hip or any non-vertebral fractures by one fourth, and reduce cancer incidence by about 17% and cancer mortality by about one third [107]. Furthermore, data presented by Priemel et al have pointed to a desirable minimum serum 25(OH)D levels of 75 nmol/L [108]. In another study, intestinal calcium absorption was increased by 45-65% in women when 25(OH)D levels increased from 50 to 80 nmol/L [109].

In 2007–2009, Canada reported for the first time a national survey examining serum 25(OH)D concentrations for ages 6-79 y in the Canadian Health Measures Survey (CHMS) [23, 110, 111].
Living above latitude 35ºN, most Canadians are not able to synthesize cutaneous vitamin D from sunlight exposure for 4-5 months of the year [89]. Overall, more than one third of Canadians not taking vitamin D supplements had 25(OH)D concentrations < 50 nmol/L in winter. The proportion of nonwhite Canadians with 25(OH)D concentrations < 50 nmol/L increased to two-thirds due to their darker skin pigmentation, requiring longer time to synthesize vitamin D. Furthermore, the prevalence of vitamin D deficiency defined as serum 25(OH)D < 30 nmol/L for white Canadians was 7%, versus 20% for nonwhite Canadians in the winter months. This translates into 1 in 5 nonwhite Canadians being at risk for rickets or osteomalacia. In addition, previous smaller studies have also examined vitamin D status of Canadians with diverse ethnic backgrounds. Gozdzik et al examined the vitamin D status of young Canadian adults in Toronto (latitude 43ºN) and clearly showed that there was a substantial variation in serum 25(OH)D concentrations according to ancestry; 34.4% of European subjects had serum 25(OH)D concentrations below 50 nmol/L, while 85.2% of East Asians and 93.5% of South Asians had levels below 50 nmol/L during the winter months [105]. Similarly, Vieth et al assessed vitamin D status of young women living in Toronto and found that 21% of white women, 31.9 % of nonwhite women (First Nations, South Asians, Indo-Asians, East Asians) and 25% of black women had serum concentrations < 40nmol/L during the winter months [112]. Together, these findings demonstrate that wintertime concentrations of serum 25(OH)D below 50 nmol/L is common in the Canadian population, particularly those in diverse ethnic communities due to increased melanin in skin interfering with endogenous vitamin D production. This is not surprising given that there is insufficient UVB for endogenous synthesis of vitamin D during winter months, as well as inadequate dietary sources of vitamin D.

2.8 Toxicity of Vitamin D

Table 2.2 shows the updated Tolerable Upper Intake Level of vitamin D for all life stage groups. The UL was revised upwards to 4,000 IU/d in individuals over the age 9y, with lower levels for younger children and 1,000 IU/d in infants up to 6 months. This tolerable upper intake level for vitamin D is intended to specify the level above which the risk of harm may increase, and is defined as the highest chronic daily intake shown to have no harmful effect. The UL serves as a measure for chronic intake of a free-living, unmonitored population. Vitamin D₃ intakes of 4,000 IU/d [113], 10,000 IU/d [114], and 40,000 IU/d [12] have been shown to be safe in previous
studies. From a physiologic perspective, an adult in a bathing suit exposed to one erythemal dose of ultraviolet radiation (a slight pinkness in skin that occurs 24 h after UV radiation) is reported to be equivalent to ingesting between 10,000-25,000 IU of vitamin D orally [30]. Healthy farmers in Puerto Rico and lifeguards in St. Louis had mean serum 25(OH)D concentrations well in excess of 130 nmol/L [115]. Serum 25(OH)D concentrations ≤220 nmol/L is considered the higher end within the physiologic range for humans that is regarded as being safe [115].

The classic form of vitamin D toxicity is hypercalcemia, although the first clinical sign of vitamin D intoxication is hypercalciuria [115]. Normally in adults, the excessive absorption of calcium is compensated for by excretion of calcium into urine. Once the renal calcium excretion can no longer balance the input from the gut then serum calcium will start to increase, hence leading to hypercalcemia. The signs and symptoms of vitamin D toxicity are due to the consequences of hypercalcemia and the subsequent dehydration. Symptoms have been reported to include weakness and fatigue, nausea, vomiting, constipation, confusion, drowsiness, and difficulty in concentration. Chronic excessive intakes of vitamin D can lead to kidney damage, and soft tissue calcification. Furthermore, most cases of vitamin D toxicity have involved vitamin D₂. The lowest intake of vitamin D associated with hypercalcemia has been with a dose of vitamin D₂ of at least 40,000 IU/d for several months [115]. There are no published cases of vitamin D intoxication at long-term doses up to 40,000 IU/d [115]. To date, the case for vitamin D₃ toxicity has been mainly as a result of industrial-scale mishaps that resulted in hypercalcemia [116-119]. In addition, patients given regular bolus doses of 300,000 IU vitamin D₃/wk have been reported to clinically manifest hypercalcemia with serum 25(OH)D levels >1000 nmol/L [120]. The average 25(OH)D concentrations in serum of confirmed cases for vitamin D toxicity has been reported to be 560 nmol/L [121], yet serum levels as low as 355 nmol/L have been also associated with hypercalcemia [122].
Table 2-2 Tolerable Upper Intake Level of Vitamin D

<table>
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<td>Infants</td>
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<tr>
<td>0 to 6 mo</td>
<td>1,000 IU (25 μg)</td>
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<tr>
<td>6 to 12 mo</td>
<td>1,500 IU (38 μg)</td>
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<tr>
<td>Children</td>
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<td>1–3 y</td>
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<td>4–8 y</td>
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<td>Males</td>
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IU= International Unit. Table adapted from reference [22].

Vitamin D toxicity is thought to occur largely when the binding capacity of DBP is exceeded, where “free” 1,25(OH)₂D is more accessible to cells [115, 117] and starts to accumulate in the adipose and muscle tissues for vitamin D [123, 124]. The mechanism of vitamin D intoxication could also be potentially explained by the level of 1α-hydroxylase enzyme activity, and the capacity to clear vitamin D metabolites from the body [125]. There are no reported case of vitamin D toxicity due to sun exposure, because vitamin D production in the skin is self-limiting.
In the context of oral intake, long-term, regular intake amounts of beyond 40,000 IU/d could cause hypercalcemia. The newly established UL of 4000 IU/d has a large margin of safety. Factors that predispose an individual to vitamin D intoxication include unregulated 25(OH)D-1-hydroxylase that occurs in granulomatous disease [127], reduced renal function [115], and primary hyperparathyroidism where the breakdown enzyme 24-hydroxylase is suppressed but 25(OH)D-1-hydroxylase is stimulated resulting in an abnormal increase in 1,25(OH)\textsubscript{2}D levels [128].
2.9 Food Fortification

2.10 Food Fortification

Food fortification refers to the deliberate addition of vitamins and minerals to foods. The benefits of food fortification are potentially large where it can be a very cost effective public health intervention. Clearly, the public health significance of the potential benefits of food fortification is primarily a function of the extent of the public health problem. Generally speaking, therefore, when deciding to implement a fortification program, priority should be given to controlling those nutrient deficiencies that are most common in the population and that have the greatest adverse effect on health and function. Moreover, the public health impact of food fortification depends on a number of factors such as the level of fortification, the bioavailability of the fortificants, and the amount of fortified foods consumed [129]. As well, it is preferable to use food vehicles that are centrally processed and to have the support of the food industry.

Food fortification has a long history of use in industrialized countries for the successful control of deficiencies of vitamins A and D, several B vitamins (thiamine, riboflavin and niacin), iodine and iron [129]. Salt iodization was introduced in the early 1920s in both Switzerland and the United States of America and has since expanded progressively all over the world to the extent that iodized salt is now used in most countries. From the early 1940s onwards, fortification of cereal products with thiamine, riboflavin and niacin became a common practice. Margarine was fortified with vitamin A in Denmark and milk with vitamin D in the United States [129].

There are different types of food fortification. These include mass fortification of foods that are widely consumed by the general population, targeted fortification of foods designed for specific population subgroups, and voluntary fortification which allow food manufacturers to voluntary fortify foods available in the market place (i.e market driven fortification) [129]. Mass fortification is almost always mandatory, targeted fortification is either mandatory or voluntary, and market-driven fortification is always voluntary but controlled by regulatory agencies. Mandatory fortification provides a higher level of certainty for delivering a sustained source of fortified food for consumption by the relevant population group, which in turn offers a public health benefit. Mandatory fortification is usually prompted by evidence that a given population is deficient or inadequately nourished, such as clinical or biochemical signs of deficiency and/or
unacceptably low levels of micronutrient intake. Voluntary fortification tends to be used when there are lower order risks to public health, i.e. when the risks to public health are not as serious or demonstrable so as to warrant mass fortification. For any given population group, there are 5 key factors that determine whether mandatory or voluntary fortification is likely the most appropriate option. In brief, they are: the severity of the problem and its prevalence within a population group, size and scale of the food industry sector responsible for the production of the proposed food vehicle, the level of knowledge among the population about the nutritional needs, the acceptable level of government intervention, and relative contribution of certain foods to the diet of the population [129].

2.11 Advantages and Limitations of Food Fortification

Food fortification offers several advantages over supplementation strategy to mitigate micronutrient malnutrition. Despite the advantages, there are also several drawbacks to fortification strategy. The general discussion of the advantages and limitations of food fortification I describe here are adapted from reference [129]. Generally, fortification of appropriate dietary sources will reach a wider segment of the population, which has the potential to improve the nutritional status of the population, both poor and wealthy. Some fortified foods may not be consumed by everyone in the population, but with mandatory fortification, the general population is exposed to increased levels of micronutrients in food, regardless of whether or not they will benefit from fortification. Food fortification does not require changes in existing food patterns or individual compliance. However, issues particularly with regard to appropriate levels of nutrients, stability of fortificants, physical properties, nutrient interactions, cooking properties and acceptable taste by consumers need to be fully resolved before implementation. Fortification is often more cost effective, especially if the technology and an appropriate food distribution system is in place. Nevertheless, there might be significant costs associated with the fortification process such as start-up costs, quality control costs which might limit the implementation and effectiveness of food fortification programs. In addition, fortified foods can fail to reach the poorest segments of the population who are at the greatest risk of micronutrient deficiency due to low purchasing power and an underdeveloped distribution channel. This drawback also applies to supplementation strategies in poor countries. Fortification carries a minimal risk of chronic toxicity when properly regulated. Dietary supplements carry a higher
risk of micronutrient intoxication particularly with accidental overdosing. However, if many foods have the nutrient, fortification can carry a risk of excess intakes. It is feasible to fortify foods with several micronutrients simultaneously without a difference in the price of the product at the point of manufacture.

Moreover, a sustained supply of micronutrients will maintain body stores of nutrients more efficiently and more effectively than will intermittent supplementation. This is an important advantage to growing children and pregnant and lactating women who are in greatest need for sustained and adequate supply of micronutrients. However, generally food fortification tends to have a less immediate impact than supplementation for acute treatment of deficiency in malnourished subpopulations. There are several concerns over fortification by consumers which include the effect on the price and taste of fortified food, proper labeling, risk of excessive intakes, and the source of the added nutrients. While supplements are an effective method for individuals to increase their micronutrient intake, food fortification represents the best opportunity to increase micronutrient supply to the population.

2.12 Vitamin D Fortification of Food

During the First and Second World Wars, fortification of food became widespread in order to prevent micronutrient deficiencies and to address nutrient losses during food processing. Fortification of food with vitamin D began in response to the development of rickets in children in industrialized countries. In 1932, soon after the structure of vitamin D$_2$ was determined, the addition of vitamin D to milk at a level of 400 IU/L in Canada and United States became largely responsible for successful eradication of vitamin D deficiency rickets in children [130].

Given the limitations of our contemporary diets and non-existent sun exposure in winter, it is clear that intakes of vitamin D from foods alone are not adequate to meet the new revised DRIs. While Canada and the United States use the same DRIs for vitamin D and calcium and the same upper limits of safe intake for these nutrients, they have very different regulatory approaches to the lawful addition of vitamin D and calcium to foods [131]. Through the Canadian Food and Drug regulations, vitamin D fortification of fluid milk (35-45 IU/100 ml) and margarine (530 IU/100 g) is mandatory in Canada [131]. Other milk products including evaporated milk, powdered milk, goat’s milk and milks of plant origin (soy, rice and other grains) require vitamin
D and calcium fortification [131]. Other foods for which vitamin D addition is permitted are meal replacements, nutritional supplements, and formulated liquid diets. Addition of vitamin D to such foods are optional but may be no less than 2.5 μg (100 IU) and no more than 10 μg (400 IU) per 1,000 kcal, as long as the intended total energy intake is <2,500 kcal [131]. Fortification of some egg products is also permitted at this time, but industrial milk used in baked goods and cheeses does not need to be fortified [131]. The approach to vitamin D fortification of foods with vitamin D in the United States is also very carefully regulated, but the lawful addition of vitamin D to eligible foods in the US is largely voluntary. Fluid milk, breakfast cereals, rice, corn meal, noodles, macaroni, yogurt, margarine, and calcium fortified fruit juices are fortified with vitamin D in the range of 40-140IU/serving [131]. Although many foods are eligible for vitamin D fortification, to this day there is still a large gap between the number of eligible foods and the number and variety of vitamin D-fortified foods observed in the marketplace. Fluid milk and ready-to-eat cereals are the major contributors to vitamin D intake in the United States, while milk and margarine are the major fortified foods contributing to Canadian vitamin D intake [132].

2.13 Rationale for Increased Vitamin D fortification

Among the strategies for increasing dietary consumption of vitamin D in the general population are: (1) increasing the intake of foods that naturally contain vitamin D; (2) defining optimal sun exposure with minimal risk of skin cancer; (3) vitamin D supplementation; or (4) increased mandatory or voluntary vitamin D fortification of food sources. Each of these strategies has its own advantages and drawbacks, but with the newly established DRIs for vitamin D, increasing the number and variety of fortified foods is the optimal solution at this point to effectively increase vitamin D intakes in North American populations during times of inadequate UVB exposure.

Food fortification has broad implications for national food policies [133]. As stated in section 2.6, the average Canadian diet provides only 200 IU vitamin D/d, falling severely short of the latest recommendation guidelines established by the IOM [22]. Since the vitamin D recommendation for children and adults has been tripled, acquiring the 600 IU/d RDA by diet alone is extremely challenging, given the existing inadequate food supply of vitamin D available for the general population. Unless fortification with vitamin D were to increase dramatically, the
need for supplementation will remain necessary. However, the major problem with supplement use is compliance, which can lead to ineffective impact in the population. Analyses of the NHANES III data demonstrated that significant racial differences have been observed for daily vitamin D supplement use and the benefits gained from vitamin D supplementation on the prevalence of hypovitaminosis D are not uniform across all age, race/ethnic, and gender groups [131, 134, 135]. Therefore, advising the population to take a vitamin D supplement is not a realistic strategy for making a significant contribution across the broader population.

Considerations should be given to changing food system in Canada to increase fortified food options for men and women of all ages and race/ethnic groups in accordance with the newly revised DRIs for vitamin D. Food fortification does not require individual compliance or changes in purchasing patterns, and would be more effective across a broader population. In consideration of food fortification with vitamin D, one must address the benefit/risk of increased vitamin D intakes in the population. To determine benefit, one must ensure that vitamin D is bioavailable from fortified foods and that it has a sizable effect in at least one population subgroup. Bioavailability is the degree to which food nutrients are available for absorption and utilization in the body. A systemic review on efficacy of food fortification showed that foods fortified with vitamin D increase circulating serum 25(OH)D concentrations in a dose dependent manner [136, 137]. The Finnish experience over the past 10 years is a good example with respect to implementation and evaluation of vitamin D fortification policies. The Finnish government initiated optional vitamin D₃ fortification of milk and yogurt at 20 IU/100g and margarine at 400IU/100g. The implementation of voluntary fortification successfully resulted in a 50% increase in mean serum 25(OH)D during the winter months and the prevalence of levels <40nmol/L decreased by 55% within a year in a subgroup sample of Finnish men between 18-28 y [138].

Another point to consider in food fortification is the nature of food vehicle to deliver vitamin D. For instance, should food sources that are naturally rich or fortified with calcium be the only targets for vitamin D fortification. Since the revised vitamin D recommendations by IOM were based solely on bone health, then implementation of increased food fortification policy will be pertinent to staple foods that deliver these two nutrients together for optimal bone health. As seen from previous studies, the problem with fortifying milk is that it does not increase the vitamin D supply in non-consumers [139]. In particular, milk consumption is lower in those populations
that are at high risk vitamin D deficiency (e.g. blacks, Asians, Native Americans) due to cultural habits and higher prevalence of lactose intolerance [105, 140]. Furthermore, there is a good range of evidence that vitamin D is bioavailable when added to a wider range of foods such as orange juice [141, 142], bread [26, 143], and cheese [1, 29]. The study by Wagner et al. successfully demonstrated that vitamin D was equally bioavailable in cheese as a vitamin D supplement [29]. These previous findings show that extending vitamin D₃ fortification to variety of foods is a viable alternative to vitamin D supplementation.

Given the safety of vitamin D and current data showing a significant large gap between dietary intakes and food supply for vitamin D, there is a need to expand vitamin D fortification to a wider range of foods with the goal of increasing vitamin D intakes in the general population including high risk groups. Health Canada will need to re-evaluate their fortification regulations in response to the revised vitamin D recommendations and the current dietary vitamin D intakes of Canadians.

2.14 Rationale for Vitamin D₃ Fortification of Cheese

With current dairy industry practices in Canada, cheese does not contain vitamin D, yet it is an excellent candidate for vitamin D₃ fortification. For my thesis research project, we chose to fortify full-fat Cheddar and Mozzarella cheeses with vitamin D₃ for the following reasons:

*Consumption:* Cheese is widely consumed around the world, its per capita consumption in Canada has increased by 44 percent between 1984 and 2003 [144]. According to “Canadian Food Trends to 2020” cheese consumption is expected to increase nearly 30 percent by 2020, yet milk consumption is expected to decline by 15 percent during 1984 and 2020 [144]. The forecasted increase in cheese sales is mainly due to the popularity of Italian, Mediterranean, and Mexican styles of cooking and from sales of cheese-containing foods such as pizza.

*Nutritional value of cheese:* cheese makes a significant dietary contribution to the intakes of essential nutrients such as calcium, phosphorus, and protein. Cheese also provides appreciable amounts of vitamin A, vitamin B₁₂, vitamin B₂, folate, as well as minerals including iodine, magnesium and zinc [145].
Health benefits: Cheese provides nutrients that are needed for good bone health. Cheese is known to contain specific factors shown to protect against dental caries [145]. There are several mechanisms that have been suggested to explain the anticariogenic effect of cheese. Studies have found that consuming cheese either alone or as part of a cooked, mixed meal, increases the concentration of calcium in the plaque around our teeth. This helps to reduce and replace the calcium lost when teeth come under attack from acids produced in the mouth. It has been shown that eating cheese after repeated consumption of acidic drinks can return the levels of calcium in the plaque to pre-drink levels [145]. In addition, cheese has low lactose content and is well tolerated by those who suffer from lactose maldigestion while providing them with the nutrients in dairy foods. Therefore, cheese is one of the most versatile, economical, and nutritious food consumed by a wide range of individuals in the general population.

Previous studies: fortification of lipid-soluble vitamin D in cheese matrix is possible, as seen from previous research. The incorporation of vitamin D in a cheese matrix has been studied by many different groups of researchers. The first study was reported by Banville et al who compared different methods for fortifying Cheddar cheese with vitamin D₃ via liposomes, emulsions, or cream solutions [146]. Kazmi et al. later evaluated the retention of vitamin D₃ in a lab-scale cheddar cheese using an emulsion solution or crystalline vitamin D₃ [147]. Following Kazmi et al., our research group demonstrated that industrially made cheddar cheeses either full-fat or low fat, were suitable foods for vitamin D₃ fortification [1]. Wagner et al. also showed that the increase in serum 25(OH)D concentrations with cheddar cheese was equivalent to that with supplementation, suggesting that the strong binding affinities of β-lactoglobulin and β-casein for vitamin D did not affect bioavailability of vitamin D from cheese [29]. Most of these previous research studies have used pre-concentrate solutions of vitamin D₃ blended with synthetic (i.e. polysorbate 80) food grade emulsifiers. Although these previous studies have shown that vitamin D can be retained in cheese, there was a partial loss of vitamin D with the milk fat in whey. Specifically the data by Wagner et al. showed that about 10% and 50% of the vitamin D added was lost into whey in full-fat and low-fat cheddar cheeses respectively, reducing the practicality of the methodology, especially with the low-fat cheddar cheese. Loss of vitamin D during manufacture could make the final fortification product unreliable. Another limitation is that the amount of vitamin D₃ used for fortification exceeded the permitted levels of vitamin D in foods. The Dairy Farmers of Canada continues to be interested in fortifying cheese with vitamin D, but
they have concerns about loss of vitamin D into whey byproduct. Hence given the opportunities, our goals are to both develop a new and improved protocol to fortify cheese with vitamin D₃, as well as to test the bioavailability and efficacy of vitamin D₃ fortification in cheese, particularly Cheddar and Mozzarella cheeses.

2.15 Rationale for Binding of Vitamin D₃ to Caseins

Complete retention of vitamin D in cheese is difficult due to partial loss of vitamin D into whey, as seen from previous research. From the study done by Wagner et al., the resulting vitamin D retention obtained in the low-fat Cheddar cheese indicated that vitamin D from the emulsion might have been taken up by the casein proteins in the cheese curd matrix which is the main constituent of low fat cheese. Furthermore, previous studies have indicated that vitamin D₃ was bound to hydrophobic portions of both β-casein and β-lactoglobulin [148]. Forrest et al proposed that milk proteins could serve as efficient delivery systems for vitamin D for the manufacture of cheese [148]. They suggested the need for further research to make binding of vitamin D to milk proteins a practical application in the dairy industry. Hence, fortification method utilizing properties of dairy proteins might be able to improve retention in cheese by protecting loss of vitamin D into whey. Few studies have investigated the binding of vitamin D to dairy proteins. Milk proteins consist of 80% caseins (αₛ₁, αₛ₂, β, κ) and 20% whey proteins, which are mainly β-lactoglobulin, α-lactalbumin, and bovine serum albumin [149]. Caseins are phosphoproteins, which are amphiphilic molecules (have areas that are hydrophobic and hydrophilic) [150]. When the various caseins interact with each other they form casein micelles, which are a system of particles of colloidal size held together and organized by means of non-covalent intermolecular binding interactions. Micelles are formed via calcium phosphate bridges of multiple submicelles, which are spherical aggregates of several casein monomers connected together by hydrophobic and electrostatic interactions [151]. The hydrophilic areas of the casein submicelles are orientated towards the exterior, with κ-caseins located at the periphery as they have a hydrophilic C-terminal end interacting with the continuous aqueous phase. The multiple κ-caseins form a “hairy” layer around the micelle and give colloidal stability against calcium precipitation [150, 151]. Sodium caseinate (NaCN) and calcium caseinate (CaCN) are formed by modifying caseins through multiple steps including the acidified extraction of the casein and the solubilization of the acid casein in solutions of NaOH or Ca(OH)₂ to neutralize the precipitated casein curd [150].
Our strategy is to pre-bind vitamin D to caseins which could be used as a delivery system to maximize the full retention of vitamin D in cheese in the interest of minimizing deleterious process induced effects during the cheese making process.
Chapter 3 : Hypotheses and Objectives
3.0 Hypotheses and Objectives

3.1 Hypotheses

Food science component:

i) Milk caseins can be used as a delivery system to maximize the full retention of vitamin D₃ in fortified Cheddar and Mozzarella cheeses.

ii) Binding of vitamin D₃ by milk caseins will protect loss of vitamin D₃ in whey.

Clinical nutrition component:

i) Baking fortified Mozzarella cheese with pizza will not substantially alter its efficacy for producing a rise in serum 25(OH)D levels after consumption.

ii) Consumption of pizza baked with Mozzarella cheese fortified at 28,000 IU vitaminD₃/serving will produce an increase in serum 25(OH)D that is greater than the increase obtained with consumption of pizza baked with Mozzarella cheese fortified at 200 IU vitaminD₃/serving.

iii) Consumption of Mozzarella cheese fortified at 28000 IU vitamin D₃/serving is safe and will not adversely alter markers of bone and mineral metabolism.

3.2 Objectives

Food science component:

i) Optimize the fortification methodology by development of a new protocol that utilizes the binding of vitamin D₃ to milk caseins.

ii) Assess the stability of vitamin D₃ in casein premix during cold storage

iii) Determine the retention of vitamin D in cheese using milk caseins as a delivery system for vitamin D₃.

a) Analyze the retention of vitamin D₃ in a lab-scale cheese model system

b) Confirm the retention of vitamin D₃ obtained in cheese model system using industrial-scale Cheddar and Mozzarella cheese productions.
Clinical nutrition component:

i) To determine the effect of consuming vitamin D₃ fortified Mozzarella cheese baked with pizza on serum 25(OH)D concentrations.

ii) Compare the rise in serum 25(OH)D concentrations between groups consuming pizza baked with Mozzarella cheese fortified either at 28,000 IU/serving or 200 IU/serving.

iii) Monitor serum calcium, phosphate, creatinine, PTH, as well as urine calcium, phosphate, and creatinine.
Chapter 4: Vitamin D₃ Fortification of Industrially Made Cheddar and Mozzarella Cheeses Using Caseins as a Delivery System
4.0 Abstract

Vitamin D nutrition remains a public health concern in North American populations where there is a limited exposure to sunshine throughout the year. Achieving the new RDA of 600 IU vitamin D/d for most adults is challenging and unrealistic due to limited food sources. The main objective of this research was to establish a cheese fortification protocol that utilizes caseins as a delivery system for incorporating vitamin D₃ in cheese, with the aim of preventing loss in whey during cheese-making. We assessed and confirmed the feasibility of the fortification protocol using pilot scale Cheddar and Mozzarella cheese production. We also assessed the stability of vitamin D₃ in casein premix during cold storage over 2 months. We examined the retention of vitamin D₃ in industrially made fortified Cheddar and Mozzarella cheeses. It was found that over 90% of vitamin D₃ added to milk was retained in both Cheddar and Mozzarella cheeses, with ~8% of vitamin D₃ loss in whey. The vitamin D₃ was uniformly distributed in the cheeses. As well, vitamin D₃ concentration in the casein premix was stable over 2 months during storage at -20°C. We conclude that although using caseins as a vehicle to deliver vitamin D₃ did not result in full recovery of vitamin D₃ in cheese, use of dairy proteins is a more natural way to deliver vitamin D as a nutrient versus synthetic emulsifiers currently used in dairy industry to fortify milk.

Key Words: Vitamin D, fortification, cheese, dairy proteins, casein
4.1 Introduction

The average Canadian diet provides ~ 200 IU /d of vitamin D, falling severely short of the latest recommendation guidelines recently established by the IOM in 2011 [22-24, 93, 131]. To acquire 600IU/d through diet alone is difficult because foods that are naturally rich in vitamin D such as fatty fish and fish liver oil are not frequently consumed. Furthermore, mushrooms and egg yolks provide very low sources of vitamin D and the concentrations are often variable [152]. Through the Canadian Food and Drug regulations, vitamin D fortification of fluid milk (35-45 IU per 100 ml) and margarine (530 IU per 100 g) is mandatory in Canada [153]. Other milk products including evaporated milk, powdered milk, goat’s milk and milks of plant origin (soy, rice and other grains) require vitamin D and calcium fortification [94]. In the US, there is a voluntary vitamin D fortification of fluid milk, breakfast cereals and fruit juices (40-140 IU per serving) [131]. Despite mandatory fortification practices in Canada, most fortified foods are under-fortified and provide inadequate amounts of vitamin D [103, 112, 154]. Cross-sectional studies suggest that current fortification practices are not sufficient in preventing vitamin D insufficiency in Canada during the winter months [112]. Furthermore, milk consumption is significantly lower among ethnic populations including First Nations, Blacks, Asians, and people of Middle Eastern origin likely due to cultural dietary habits and higher prevalence of lactose intolerance [105, 106, 139, 140]. Vitamin D fortification of foods other than milk and margarine is limited in Canada. However, there has been a wide range of evidence that vitamin D is stable and bioavailable when added to orange juice [141, 142], bread [26, 143], and cheeses [1, 29, 155].

Cheese consumption is projected to increase by nearly 30 percent over the next 15 years [144]. Cheese is widely consumed around the world, and its per capita consumption in Canada increased by 44 % between 1984 and 2003 [144]. The forecasted increase in cheese sales are mainly due to the popularity of Italian, Mediterranean, and Mexican styles of cooking and much of the cheese is consumed as an ingredient on pizza and pasta dishes. Cheese is a good vehicle for vitamin D fortification [1, 29, 155]. The feasibility of vitamin D₃ fortification in full fat and low fat cheddar cheeses was previously shown, providing further evidence that vitamin D₃ is not degraded during processing, ripening for 12 months, or after thermal treatment at > 200 °C for 5 min [1]. Furthermore, fortification does not alter the yield, chemical composition or flavor of the resulting cheddar cheese [1, 155]. Although the vitamin D₃ recovery in fortified full fat cheddar
was > 90%; in low fat cheese, only 55% of the vitamin D₃ was retained with the remainder lost in the whey [1]. Given its low fat content, loss in whey suggested probable interaction of vitamin D₃ whey proteins. Previously, it has been reported that vitamin D₃ interacts with β-lactoglobulin (β-LG), a major whey protein [156, 157].

Most previous studies have used liquid preparations of vitamin D₃ dissolved in propylene glycol and polysorbate 80 to overcome vitamin D insolubility [1, 146, 147]. Given its hydrophobicity, this poses further challenges for fortification of low and nonfat food products. To overcome these challenges, previous studies have investigated interaction and stabilization of vitamin D by casein micelles [156]. Forrest et al showed that vitamin D₃ interacts with the hydrophobic core regions of β-casein [156]. Similarly, Livney et al showed that casein micelles have the ability to encapsulate vitamin D₃ via adsorption of vitamin D₃ onto the hydrophobic core regions of the casein micelles [158, 159]. Casein micelles comprise approximately 80% of the proteins in milk [156, 158-160]. These authors proposed that encapsulation of vitamin D by casein micelles could serve as an efficient delivery system of vitamin D for fortification of staple foods using natural ingredients over the commercially available food emulsifiers, and without modifying sensory properties [159] [156] [158].

Our objectives were to: i) establish a fortification protocol that utilizes binding of vitamin D₃ to caseins to incorporate vitamin D into cheese, with the aim of preventing loss in whey during cheese-making; ii) assess the feasibility of the fortification protocol for larger scale fortification of cheddar and mozzarella cheeses with vitamin D₃; and iii) confirm the stability of vitamin D₃ in a casein premix and cheese during cold storage. We examined the recovery and distribution of vitamin D₃ in pilot scale fortified Cheddar and Mozzarella cheeses.

4.2 Materials and Methods

Vitamin D₃ Casein Premix

Ethanolic Vitamin D₃ Solution

Stock vitamin D₃ solution was prepared by dissolving white crystalline vitamin D₃ (USP grade, DSM, Basel Switzerland, Sigma) in USP grade ethanol. The molar concentration of the stock vitamin D₃ solution was adjusted to 2.0 x 10⁶ IU mL⁻¹ (5.0 x 10⁴ µg mL⁻¹). Quantification of
vitamin D₃ was based on an absorbance at 265 nm (33.36 absorbance units based upon a molar extinction coefficient of 18,300 absorbance units mol⁻¹L⁻¹). Spectroscopy was performed with an 8352A diode array spectrophotometer (Hewlett-Packard) using a 1-cm quartz cuvette.

**Precipitation of Acid Caseinate**

Acid caseinate curd was precipitated from skim milk (pasteurized and homogenized, Parmalat Dairy Inc., Beatrice skim milk). The pH of the skim milk was decreased from its initial pH of 6.8 at 20°C, by gradually adding ~150 mL of citric acid solution (0.3 mol L⁻¹, monohydrate crystalline, USP grade, Fisher Scientific) to 3 L skim milk under constant stirring, until the isoelectric point was reached at pH ~ 4.6. The acidified milk mixture was then heated by increasing the heat temperature from 20°C to 50-55°C. As a result, the caseins precipitated as very fine particles in a liquid serum (whey). The pH of whey at separation was ~4.5. After a brief period of cooling, the precipitated caseins were separated from the whey, by filtering the casein curd agglomerates through a cheese cloth. The casein curd was then air dried at ambient temperature overnight (20-25 °C). Through this acidification process, the starting skim milk (3 L) yielded ~ 0.23 kg acid caseinate curd [7.7% (w/w)].

**Preparation of Vitamin D₃ concentrated Casein Premix**

The precipitated acid caseinate curd (0.15 kg) was blended with distilled water (0.3 kg) to yield ~33% (w/w) casein suspension. The suspension was thoroughly blended using a Polytron mixer (Kinematica CH-6010 KRIENS-LU), until very fine particles of casein suspension were evident. Food grade monobasic sodium phosphate (0.6 g) (Quadra Chemicals LTD, Burlington, Ontario) was added to casein suspension and blended for additional 5 minutes with the polytron mixer. The molar sodium phosphate concentration of the casein suspension was 10 mmol/L. The casein suspension was light cream in color, and free from casein curd agglomerates. The initial pH of the casein suspension was raised from 5.2 to a final pH of 6.9-7.0, by a dropwise addition of 35 mL of 1 M NaOH while under constant stirring. The casein suspension was left overnight under constant stirring at room temperature (~25 °C). After overnight stirring, 45 mL of the stock vitamin D₃ solution pre-dissolved in ethanolic solution (2.0 x 10⁶ IU mL⁻¹) was added to the acid caseinate suspension. The final vitamin D₃ concentration was adjusted to ~185,000 – 186,000 IU mL⁻¹. Immediately following spiking, the vitamin D₃-Casein suspension was homogenized.
(APV-1000, APV, Albertstund, Denmark) at 5000 psi (35 MPa) for 5 cycles. After homogenization, the vitamin D₃ concentrated casein premix was kept at -20°C for storage and stirred thoroughly before usage.

**Vitamin D₃ fortification procedure**

*Fortification of Milk*

The fortification of milks destined for industrial scale cheese manufacturing was carried out at Agropur Research and Development Centre (ARDC) (Saint-Hubert, Quebec, Canada). Raw whole milks were obtained from an Agropur dairy farm and delivered to the ARDC. The starting raw milks were nonhomogenized, unfortified, and unpasteurized. Fortification of raw whole milks destined for cheddar and mozzarella cheeses were carried out separately on the day of the cheese manufacture.

The amount of vitamin D₃ concentrated casein premix added to raw whole milks destined for either Cheddar or Mozzarella cheese was based on (i) the preliminary findings for vitamin D₃ retention in a small cheese model system (~90% for Cheddar cheese and ~70% for Mozzarella cheese (w/w)); (ii) the targeted vitamin D₃ dose in the cheeses (i.e., 200 IU and 28000 IU vitamin D₃ per 28 g serving, both for Cheddar and Mozzarella cheeses), and (iii) the expected yield in the industrially made cheeses (~10% for Cheddar cheese and ~14.5% for Mozzarella cheese).

*Cheese Manufacture.*

All cheeses were manufactured by Agropur at the ARDC using industrial cheesemaking methodologies. Two different batches of cheese (low dose; 200 IU/serving, and high dose; 28000 IU/serving) were made for both Mozzarella and Cheddar cheeses.

*Mozzarella Cheese*

Nonhomogenized raw whole milk [3.2% fat (w/w)] fortified with vitamin D₃ was pasteurized at 74°C for 16 s prior to cheesemaking. After pasteurization, 450 kg of the pasteurized whole milk was collected at 35 °C and 67.5 ml of calcium chloride solution (0.015% w/w) was added. The cheesemilk (pH 6.5; 35°C) was then inoculated with 46.8 g of bacterial culture (Chr Hansen Easy set, 0.104g/kg, Agropur collection). After 45 min of ripening at 35°C under constant stirring, 31.5 mL of rennet [0.007% (w/w), Fromase XLG (DSM Food Specialties)] was added and
stirred. Coagulation, pH, and temperature were monitored throughout the process. The curd was cut 25 min after renneting, stirred gently for 15 min, and heated from 35 to 40 ºC during 20 min. When the pH of the whey dropped from 6.4 to 6.2, the whey was drawn and the curd was collected. The collected curd was cut into smaller curd blocks and flipped every 15 min for a total of 5 times until the pH reached 5.25, after which the curd was milled. The milled curd was then passed through a cooker containing hot water (65ºC), where the curd passing through the hot water was heated from 38 to 53.5 ºC. The cooking step causes the stretching of the milled cheese curd, which gives mozzarella cheese its unique texture. After passing through the cooker (5min), the stretched cheese curds were transferred into rectangular molds. The molded cheese curds were cooled and brined at 4 ºC for a total of 4hrs. The brined cheese was vacuum-packaged and stored in a refrigerator at 4 ºC.

**Cheddar Cheese**

Nonhomogenized, vitamin D₃ fortified raw cheesemilk [4.0% fat (w/w)] was pasteurized at 74 ºC for 16 s, and 450 kg of pasteurized cheese milks were collected at 32 ºC, after which 90.0 mL of calcium chloride solution [0.02% (w/w)] and 90.0 mL of Annatto color [0.02% (w/w)] were added. The cheesemilk (pH 6.6; 32 ºC) was then inoculated with 0.90 kg of mesophilic starter culture [0.20% (w/w), Agropur collection]. After 45 min of ripening at 32 ºC under constant stirring, 31.5 mL of rennet [0.007% (w/w), Maxiren (DSM Food Specialties)] was added to the mixture and stirred. Coagulation, pH, and temperature were monitored throughout the rennetting process. The average gelling time was 13 min (pH 5.45-6.55). The curd was cut 30 min after rennetting, stirred gently for 15 min, and heated from 32 to 39ºC in 30 min (0.23 ºC/min). The curd was then stirred gently at 39 ºC until the pH was 6.3, after which 50 kg of whey was pre-drawn and replaced with 50 kg of water (35 ºC). The curd was continued to be stirred gently at 38 ºC until the target pH of 6.25 was reached, after which the whey was drawn. The curd was then divided into two parts and baked in the vat at 37.5 ºC (cheddaring). During cheddaring process, the cheese was cut into smaller curd blocks and turned every 15 minutes until final pH of 5.35 was reached. The curd was then milled, salted [2.75% (w/w)], agitated for 5 min, wrapped in cheesecloth, and transferred into rectangular molds. Each mold contained ~ 12.5 kg of curds. The salted curd was pressed at room temperature at an applied pressure of 20 psi for 20 min, then at 30 psi for 1 h, and finally pressed at 40 psi overnight. After pressing, the pressed
whey was drawn. The pressed cheeses were vacuum-packaged and stored in a refrigerator at 4 °C.

Analysis

**Sampling of Milk, Whey, and Cheese for Vitamin D₃ Analysis**

Samples of fluid milk, whey, cooking water, and cheeses were saponified and extracted using the method of Bligh and Dyer lipid extraction according to the method published by Wagner et al.[1], but in our method we used an external standard in place of an internal standard for vitamin D₃ quantification. Vitamin D₃ extractions were carried out only for the high dose (28000 IU per serving) Cheddar and Mozzarella cheese samples. Due to methodological complications associated with separation and detecting low vitamin D levels by UV absorption in the low dose (200 IU per serving) cheese samples, vitamin D₃ content in the low dose cheeses was estimated based on (i) the amount of vitamin D₃ added to starting cheese milks; (ii) calculated vitamin D₃ retention (%) in the high dose cheese samples.

Samples (1 g) of fortified cheesemilks and wheys for the high dose Cheddar and Mozzarella were used as for the saponification and extraction of vitamin D₃. For the high dose Cheddar and Mozzarella cheeses, random sites were drawn from 2 kg cheese blocks and shredded with a fine cheese grater; 0.2 g of the shredded cheese samples were weighed into 10 mL test tubes and mixed with 0.8 mL of distilled water.

**Analysis of Vitamin D₃ Content**

Samples (1g) of cheesemilk and whey were heat saponified with 0.5 mL of aqueous potassium hydroxide (KOH, 60% w/v), and the lipids were extracted with methanol/chloroform according to the method of Wagner et al. [1]. Vitamin D₃ was quantified using an Agilent 1100 series isocratic HPLC, equipped with detectors set at 266 and 228 nm. Absorbance data were recorded to a computer, and ChemStations software (Agilent, Mississauga, Ontario, Canada) was used to integrate the peak areas. The concentration of vitamin D₃ in the extracted samples were determined by established instrument calibration for known concentrations of vitamin D₃ (25-100 µg mL⁻¹) and a reference vitamin D₃ external standard in mobile phase (25 µg mL⁻¹). A Zorbax Eclipse XDB-C₁₈ HPLC column (4.6 x 150 mm, 5 µm, Agilent Technologies) was used. Operating conditions were ambient temperature (21 °C); methanol/water mobile phase (95:5
v/v); isocratic flow rate of 1.0 mL min$^{-1}$; and an injection volume of 50 µL. The peak heights absorbed at both 266 and 228 nm confirmed the presence of vitamin D$_3$ with twice as large peak area absorbed at 266 nm compared to 228 nm. The area under the curve at 266 nm (maximal absorbance wavelength) for vitamin D$_3$ external standard was used as a reference index of vitamin D$_3$ concentrations in the samples.

**Chemical Composition of Milks, Wheys and Cheeses**

Chemical composition and microbiological analyses were carried out by Agropur at their accredited Central Analysis Laboratory (Granby, Quebec, Canada). Chemical composition of the cheeses (fat, protein, moisture, salts, total solids) was performed using a Food Scan Analyzer (Foss Analytical A/S) which uses the near infrared spectra to estimate the chemical composition of the cheeses. The food scan analyzer is regularly calibrated against standard Association of Official Analytical Chemists (AOAC) methods to maintain proper calibration. For milks and wheys samples, the milk analyzer using mid infrared wavebands (2-15 µm) (Bentley instruments) was used to determine fat, protein and solid content in the milk and whey samples. The milk analyzer was calibrated against established reference laboratory methods for fat (Rose Gottleib according to ISO 1211), protein (Kjeldahl according to ISO 8968-1) and solids (FIL-IDF 21 B). Microbial analyses in the cheeses (yeast, mold, total coliform, *Clostridium perfringens*, *Escherichia coli*, *Listeria*, *Salmonella*) were performed using standard AOAC methods [161].

**Statistical Analyses**

The results are presented as means ± SDs (unless indicated otherwise). All data were analyzed with SPSS software (version 13.0). Between group differences in the yields, chemical composition, and recoveries of milks, wheys, and cheeses, were analyzed with one-way ANOVA and t-tests. Storage stability and distribution of vitamin D$_3$ in casein premix and fortified Mozzarella cheese were analyzed using repeated measures ANOVA. The criterion for statistical significance was set at P < 0.05.
4.3 Results and Discussion

*Milk, Whey, and Cheese Composition*

Chemical compositions of cheesemilks and wheys for the low (200IU/serving) and high (28000IU/serving) dose cheeses are shown in Table 4.1. The fortification level of vitamin D$_3$ did not substantially change the fat, protein, and solid contents of either the Cheddar and Mozzarella cheesemilks or wheys. The chemical compositions of Cheddar and Mozzarella milks and wheys differed only slightly. Even though our fortification protocol used caseins as a vehicle to deliver vitamin D$_3$, our findings were similar to those of Wagner et al. [1], who found no differences in the chemical composition of unfortified and fortified Cheddar cheesemilks and wheys using a conventional vitamin D$_3$ emulsion that is routinely used in the dairy industry to fortify milk.

**Table 4-1 Chemical Composition of Milks and Wheys**

<table>
<thead>
<tr>
<th></th>
<th>Cheddar cheese (200 IU)</th>
<th>Cheddar cheese (28000 IU)</th>
<th>Mozzarella cheese (200 IU)</th>
<th>Mozzarella cheese (28000 IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (kg)</td>
<td>43.3</td>
<td>44.1</td>
<td>41.0</td>
<td>46.9</td>
</tr>
<tr>
<td>[% yield]</td>
<td>[9.62]</td>
<td>[9.80]</td>
<td>[15.2]</td>
<td>[15.6]</td>
</tr>
<tr>
<td>Fat (%w/w)</td>
<td>33.6 ± 0.12$^a$</td>
<td>33.7 ± 0.21$^a$</td>
<td>22.9 ± 0.10$^b$</td>
<td>21.7 ± 0.09$^c$</td>
</tr>
<tr>
<td>[% recovery]</td>
<td>[80.0]</td>
<td>[82.1]</td>
<td>[87.7]</td>
<td>[90.8]</td>
</tr>
<tr>
<td>Protein (%w/w)</td>
<td>25.4 ± 0.26$^a$</td>
<td>25.4 ± 0.34$^a$</td>
<td>25.9 ± 0.20$^b$</td>
<td>24.5 ± 0.36$^c$</td>
</tr>
<tr>
<td>[% recovery]</td>
<td>[73.7]</td>
<td>[75.1]</td>
<td>[77.4]</td>
<td>[78.3]</td>
</tr>
<tr>
<td>Solids (%w/w)</td>
<td>64.5 ± 0.33$^a$</td>
<td>64.0 ± 0.27$^{ab}$</td>
<td>54.0 ± 0.19$^b$</td>
<td>51.6 ± 0.30$^c$</td>
</tr>
<tr>
<td>[% recovery]</td>
<td>[56.1]</td>
<td>[56.2]</td>
<td>[54.5]</td>
<td>[54.2]</td>
</tr>
<tr>
<td>Moisture (%w/w)</td>
<td>35.5 ± 0.33$^a$</td>
<td>36.0 ± 0.27$^{ab}$</td>
<td>46.0 ± 0.19$^b$</td>
<td>48.4 ± 0.30$^c$</td>
</tr>
<tr>
<td>Salt (%w/w)</td>
<td>1.98 ± 0.06$^a$</td>
<td>1.82 ± 0.16$^{ab}$</td>
<td>1.23 ± 0.11$^b$</td>
<td>1.35 ± 0.12$^b$</td>
</tr>
</tbody>
</table>

Mean of 8 replicates. Mean ± standard deviation. Means of measures between the two doses of vitamin D$_3$ fortified Cheddar and Mozzarella cheeses with different letters differ, (p < 0.05). Statistical testing not performed on singleton measures, indicated by the absence of a letter.

The yields, recoveries, and chemical compositions of fortified Cheddar and Mozzarella cheeses are shown in Table 4.2. As is expected in industrial cheesemaking, chemical compositions between Cheddar and Mozzarella cheeses differed significantly (P < 0.05). Solids, moisture and salt contents differed significantly between the low and high dose Cheddar cheeses (P < 0.05). As well, the chemical compositions in the Mozzarella cheeses differed significantly except for
the salt content (P < 0.05). According to the results reported by Wagner et al. [1], vitamin D₃ fortification did not affect the yields, recoveries, or chemical compositions of the fortified cheddar cheeses. Since we did not manufacture unfortified Cheddar and Mozzarella cheeses to serve as a control comparison, it is difficult to specifically determine whether the level of vitamin D₃ fortification affected the chemical composition of the cheeses. These observed differences in the chemical compositions were most likely due to sampling and methodological variance rather than an effect of vitamin D₃ fortification process, because milligram amounts of vitamin D₃ could not have altered the chemical compositions of the cheeses obtained in this study.

**Table 4-2 Yields, Recoveries, and Chemical Composition of Fortified Cheeses**

<table>
<thead>
<tr>
<th></th>
<th>Mass (Kg)</th>
<th>Fat (%w/w)</th>
<th>Protein (%w/w)</th>
<th>Solids (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar fortified milk</td>
<td>(550); [550]</td>
<td>(4.04); [4.03]</td>
<td>(3.32); [3.32]</td>
<td>(12.6); [12.6]</td>
</tr>
<tr>
<td>(200 IU); [28000 IU]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheddar total whey</td>
<td>(449); [447]</td>
<td>(0.58); [0.57]</td>
<td>(0.79); [0.79]</td>
<td>(6.20); [6.22]</td>
</tr>
<tr>
<td>(200 IU); [28000 IU]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheddar pressed whey</td>
<td>(4.62); [4.66]</td>
<td>(8.37); [6.69]</td>
<td>(1.41); [1.24]</td>
<td>(12.6); [11.2]</td>
</tr>
<tr>
<td>(200 IU); [28000 IU]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mozzarella milk</td>
<td>(450); [450]</td>
<td>(3.23); [3.13]</td>
<td>(3.94); [3.98]</td>
<td>(12.2); [12.2]</td>
</tr>
<tr>
<td>(200 IU); [28000 IU]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mozzarella total whey</td>
<td>(388); [381]</td>
<td>(0.46); [0.34]</td>
<td>(1.03); [1.02]</td>
<td>(6.60); [6.53]</td>
</tr>
<tr>
<td>(200 IU); [28000 IU]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mozzarella cooker water</td>
<td>(46.7); [46.7]</td>
<td>(0.75); [1.32]</td>
<td>(0.11); [0.20]</td>
<td>(1.35); [2.39]</td>
</tr>
<tr>
<td>(200 IU); [28000 IU]*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical testing not performed because values were measured in singleton. * Values based on estimate; high volume of water was lost during the process.

**Recovery of Vitamin D₃ in the Fortified Cheeses**

To our knowledge, this is the first reported instance of the fortification of Mozzarella cheese with vitamin D₃. Analysis of vitamin D₃ in the cheeses showed that over 90% of vitamin D₃ added to milk was retained both in Cheddar and Mozzarella cheeses (**Table 4.3 and Table 4.4**). The remaining vitamin D₃ was recovered in the whey.
Table 4-3 Vitamin D₃ Recovery in High Dose (28000 IU/serving) Cheddar cheese

<table>
<thead>
<tr>
<th>Modified Bligh &amp; Dyer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery in Cheddar cheese (%)</td>
</tr>
<tr>
<td>Recovery in whey (%)</td>
</tr>
<tr>
<td>total</td>
</tr>
<tr>
<td>pressed</td>
</tr>
<tr>
<td>Total recovery (%)</td>
</tr>
</tbody>
</table>

Recovery mean (%) ± standard deviation (mean of four replicates) of vitamin D₃ in high dose Cheddar cheese samples. Bligh & Dyer extraction; aRecovery in cheese (%) = (vitamin D₃ in cheese x kg of total cheese / vitamin D₃ in milk x kg of milk) x 100. bRecovery in whey (%) = (vitamin D₃ in whey x kg of whey / vitamin D₃ in milk x kg of milk) x 100. cTotal recovery (%) = (recovery in cheese + recovery in total whey + recovery in pressed whey).

Table 4-4 Vitamin D₃ Recovery in High Dose (28000 IU/serving) Mozzarella cheese

<table>
<thead>
<tr>
<th>Modified Bligh &amp; Dyer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery in Mozzarella cheese (%)</td>
</tr>
<tr>
<td>Recovery in total whey (%)</td>
</tr>
<tr>
<td>Recovery in cooking water (%)</td>
</tr>
<tr>
<td>Total recovery (%)</td>
</tr>
</tbody>
</table>

Recovery mean (%) ± standard deviation (mean of four replicates) of vitamin D₃ in high dose Mozzarella samples. Bligh & Dyer extraction; aRecovery in cheese (%) = (vitamin D₃ in cheese x kg of total cheese / vitamin D₃ in milk x kg of milk) x 100. bRecovery in total whey (%) = (vitamin D₃ in whey x kg of total whey / vitamin D₃ in milk x kg of milk) x 100. cRecovery in cooking water (%) = (vitamin D₃ in cooker water x kg of cooker water / vitamin D₃ in milk x kg of milk) x 100. dTotal recovery (%) = (recovery in cheese + recovery in total whey + recovery in cooking water).

Our results are in accordance with data published on vitamin D₃ fortification of small and large-scale Cheddar cheeses. Ganesan et al., Wagner et al., and Kazmi et al. all reported over 90% recoveries in Cheddar cheeses fortified with vitamin D₃ via emulsions of polysorbate 80 [1, 147, 155]. In contrast, Banville et al. reported 40-60% vitamin D₃ retention in Cheddar cheese depending on the vitamin D₃ fortification method, either using liposomes, emulsions, or cream. The authors speculated that the losses were due to vitamin D degradation during the cheesemaking process. However, our data confirm that vitamin D₃ was not destroyed during Cheddar and Mozzarella cheesemaking because we were able to recover the full amount of the vitamin D₃ added to starting milk (Milk; cheese plus whey). It is possible that the vitamin D₃
extraction technique used by Banville et al. may explain these discrepancies. Using the method of Wagner et al for both Cheddar and Mozzarella cheeses, we found that ~8% of the vitamin D₃ was lost into whey. Similarly, previous studies by Kazmi et al and Ganesan et al reported 7-10% loss of vitamin D₃ in whey from small-scale Cheddar cheese [147, 155], and Wagner et al reported 16% loss of vitamin D₃ to total whey from industrial-scale Cheddar cheese [1]. To our knowledge, this is the first study to utilize caseins as a delivery vehicle for vitamin D₃ in fortification of Cheddar and Mozzarella cheeses. Since we employed a different fortification protocol, our data are not strictly comparable to previous findings using emulsions of polysorbate 80, but could be used as a contrast. Moreover, the results reported by Wagner et al allow for a better comparison to our findings, at least for industrial scale Cheddar cheese.

Our fortification protocol had limitations. Firstly, we only produced a single batch of Cheddar and Mozzarella cheese fortified at 2 different levels, because replicating the experiment was unfeasible on an industrial-scale production. However, as a preliminary study to assess the feasibility of the fortification protocol prior to the industrial cheese manufacturing, we replicated experiments for production of lab-scale cheese model system and documented over 90% recovery of vitamin D₃ in a cheese model system (data not shown). Secondly, our extraction method was incapable of detecting the lower concentrations of vitamin D₃ in Cheddar and Mozzarella cheeses fortified to 200 IU/serving. However, the vitamin D₃ retentions for the high dose Cheddar and Mozzarella cheeses could serve as an estimate for the vitamin D₃ recoveries in the low dose Cheddar and Mozzarella cheeses, given that the cheesemaking process were identical under the same manufacturing conditions. Using the same fortification protocol, there is no reason to suspect that a lower fortification level would result in a significant change in vitamin D₃ retention.

We measured the binding efficiency of vitamin D₃ in casein protein suspension. Using an ultrafiltration technique, 85% of the vitamin D₃ remained in the casein suspension, with the remaining 15% detected in the supernatant (data not shown, analyses were duplicated). The ultrafiltration technique was an indirect index of vitamin D₃ binding to the caseins in the suspension. As our aim was to optimally deliver all of the casein-bound vitamin D into cheese manufactured from fortified milk in the interest of minimizing vitamin D loss in whey, in essence we’ve successfully delivered over 90% of the vitamin D₃ bound to casein proteins into
cheese with the remaining portion entrained in the cheese whey. Therefore, vitamin D loss into whey becomes unavoidable even when utilizing caseins as a delivery vehicle for vitamin D$_3$ for unspecified factors. Generally during cheesemaking, some fat is lost to whey [0.3-0.6 % (w/w), Table 4.1] and given its fat-soluble nature, it is likely that vitamin D will interact with the entrained fat. Loss of vitamin D was also evident in cooker water used during Mozzarella cheese manufacture, which had a 0.75-1.32 % (w/w) fat content (Table 4.1). In addition, vitamin D$_3$ may interact with $\beta$-lactoglobulin and other whey proteins [156, 157]. Furthermore, from our preliminary ultrafiltration data, ~15% of the vitamin D$_3$ was “unbound” meaning that it was not protected by caseins and could potentially either interact with fat globules or $\beta$-lactoglobulin proteins in whey. To date, there is no published mechanism on the retention of vitamin D in cheese-curd matrix. Therefore, with the current state of knowledge, it is difficult to identify whether vitamin D mainly interacts with fat globules (given its fat soluble nature) or caseins which essentially make up nearly all of the proteins retained in cheese. Moreover, it is also challenging to assess whether vitamin D retention is affected by the porosity of the rennet gel and subsequent cheese curd formation which are highly influenced by cheesemaking procedures.

Taken together with our preliminary findings, these results demonstrate that casein proteins could potentially provide protection for vitamin D$_3$ against degradation due to factors such as oxidation, acidity, light, heat, and humidity, which are all often part of cheesemaking processes [162]. The heat degradation protection was confirmed by Livney et al who reported significant protection of vitamin D$_3$ against heat (80 °C for 1 min) via encapsulation by reconstituted casein micelles [158]. Taken together with our results, these observations suggest that casein proteins have a significant potential for providing protection and stability for vitamin D$_3$ during cheesemaking process. The casein protein encapsulation mechanism of vitamin D$_3$ is outlined by Livney et al in their proposed model for the re-assembly of casein micelles, incorporating vitamin D in their core [158], and is outside the scope of this research paper.

**Stability and Distribution of Vitamin D$_3$**

The stability of vitamin D$_3$ was assessed in the casein premix and shredded mozzarella cheese during cold storage at -20°C over a short term period, given that freeze-thaw cycling may
decrease protein stability and may subsequently affect the vitamin D₃ content [163]. Table 4.5 shows the vitamin D₃ content in casein premix over 2.5 months period kept frozen at -20°C. The measured vitamin D₃ concentrations randomly sampled in the casein premix were not significantly different at any of the time points (P > 0.05). The stability of vitamin D₃ in casein premix is in accordance with the results of Livney et al. who showed the stability and protection conferred by encapsulation of vitamin D₃ in reconstituted casein micelles during storage at 4°C for a period of 28 d [158].

**Table 4.5** Stability of Vitamin D₃ in Casein Premix through Storage at -20°C

<table>
<thead>
<tr>
<th>Storage period</th>
<th>14 days</th>
<th>1.5 months</th>
<th>2.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein premix (IU/g)</td>
<td>185450 ± 4056ᵃ</td>
<td>188400 ± 574ᵃ</td>
<td>184400 ± 5971ᵃ</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (mean of four replicates). Means of measures between time points with different letters differ (P < 0.05).

**Table 4.6** shows the vitamin D₃ content of the Mozzarella cheese fortified to 28000 IU/serving that was shredded and stored at -20°C over 3-months period. Similarly, the measured vitamin D₃ concentrations sampled at random sites from shredded, fortified Mozzarella cheese were not significantly different at any of the time points (P > 0.05). The stability data of Mozzarella cheese confirm the previously reported stability of vitamin D in Cheddar cheese fortified with vitamin D₃ over short (3-5 months) [146, 147] and long (> 9 months) [1, 164] term storage periods. In addition, the random sampling sites from casein premix and shredded Mozzarella cheese at different time points showed that vitamin D₃ was evenly distributed with no significant differences in the measured vitamin D₃ concentrations. These results are similar to findings of Wagner et al and Upreti et al who also reported even distribution of vitamin D₃ in fortified cheeses [1, 164]. As expected, the even distribution of vitamin D₃ in casein premix and the readily dispersible nature of the casein premix that was added to milk under constant rigorous mixing might have further facilitated the even distribution of vitamin D₃ in cheese, which is in accordance with even distribution of fat, protein and solids in the final product.
Table 4-6 Stability and Distribution of Vitamin D₃ in Mozzarella Cheese through Storage at -20°C

<table>
<thead>
<tr>
<th>Storage period</th>
<th>14 days</th>
<th>2.5 months</th>
<th>3.5 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mozzarella cheese (IU/g)</td>
<td>1131.9 ± 31.5ᵃ</td>
<td>1117.2 ± 18.2ᵃ</td>
<td>1058.0 ± 48.8ᵃ</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (mean of three replicates). Means of measures between time points with different letters differ (p < 0.05).

4.4 Conclusion

We have demonstrated a new fortification protocol that utilizes caseins as the vehicle for vitamin D₃ fortification of industrial scale Cheddar and Mozzarella cheeses. Based on our findings, binding of vitamin D₃ to caseins did not result in full retention of vitamin D₃ either in Cheddar or Mozzarella cheese. These results demonstrate that caseins did not provide sufficient protection to prevent vitamin D loss into whey. Future research should aim to determine the mechanism of vitamin D retention in cheese, and assess the fortification of low and nonfat food products via caseins for the delivery of vitamin D. Nonetheless, caseins could be used as a delivery system for vitamin D fortification of Cheddar and Mozzarella cheeses using more natural ingredients over the synthetically emulsified form of vitamin D₃ that is routinely used in the dairy industry to fortify milk. The extension of vitamin D₃ fortification in dairy cheese products may have important implications in increasing fortified food options for Canadians who are currently consuming insufficient amounts of vitamin D from dietary sources.
Chapter 5: The Bioavailability of Vitamin D₃ Fortified Mozzarella Cheese under High Temperature Pizza Baking Process
5.0 Abstract

In the absence of adequate sun exposure, acquiring the new RDA of 600-800 IU/d for vitamin D through diet alone is difficult. There is a need to increase dietary sources of vitamin D for Canadians. Our objective was to assess the effect of pizza baked with vitamin D₃ fortified Mozzarella cheese on serum 25(OH)D concentrations. Ninety-six adults were randomized to weekly pizza meals baked with fortified Mozzarella cheese delivering either 200 IU or 28000 IU vitamin D₃ per 28 g of cheese. The primary outcome was to assess vitamin D bioavailability over 10 weeks in groups receiving either the low dose or the high dose vitamin D pizza meals, as measured by the serum 25-hydroxyvitamin D [25(OH)D] levels. Serum 25(OH)D response increased by 5.1 ± 11 nmol/L in the low dose vitamin D group [200IU/wk; N(47); P=0.003], and by 73 ± 22 nmol/L in the high dose vitamin D group [28000IU/wk; N(49); P<0.0001]. Compared with baseline, serum parathyroid hormone (PTH) decreased by 13% in the high dose vitamin D group (P= 0.008), but remained unchanged in the low dose group (P= 0.54). Our present study demonstrates that vitamin D₃ is bioavailable from fortified Mozzarella cheese baked with pizza suggesting that the high temperature baking process does not significantly breakdown vitamin D₃.
5.1 Introduction

In the last decade, there has been tremendous research in the roles of vitamin D beyond skeletal health, ranging from cancer prevention to increased immunity, possible roles in diabetes, as well as preeclampsia during pregnancy [2-4, 7, 8, 12-14, 165-167]. With a growing body of evidence, the Institute of Medicine (IOM) conducted a thorough review of all available evidence to publish a report on Dietary Reference Intakes (DRIs) for calcium and vitamin D [22]. In the assumed absence of sunlight, the IOM increased the recommended vitamin D intakes from 200 IU/d to 600 IU/d for children and most adults. Serum 25(OH)D level of approximately 50 nmol/L was set to cover 97.5% of a healthy population, recognizing only the skeletal health outcomes as relevant to dietary advice and health policy. However, controversy over optimal serum 25(OH)D levels for health maintenance beyond skeletal health outcomes remain a subject of debate. Serum 25(OH)D levels > 75 nmol/L are associated with multiple health outcomes, not only bone health [25, 93-99]. Whether health policy makers will consider the health benefits of vitamin D beyond skeletal health is not known. Vitamin D is a special case because it can be obtained through cutaneous synthesis upon exposure to UV-B sunlight, the major contributor to vitamin D status during the summer time. However, reliance on dietary sources of vitamin D is greatest in the winter at latitudes outside the tropics (>30°N and >30°S) and among people with restricted skin sunshine exposure [101, 102]. Living above latitude 35°N, most Canadians are not able to synthesize cutaneous vitamin D from sunlight exposure for 4-5 months of the year [89]. Furthermore, season, time of the day, cloud cover, smog, skin pigmentation, aging, clothing, sunscreen use are all factors that can affect the amount of UVB radiation reaching skin surface and vitamin D synthesis [153, 168-172]. Taking these factors into account, most Canadians are heavily reliant on dietary sources of vitamin D. The typical Canadian diet provides insufficient vitamin D, mainly due to few natural food sources of vitamin D. The average Canadian diet provides 200 IU vitamin D/d (5.0 µg/d), falling severely short of the new RDA intakes for most adults [23, 24, 93, 131]. There is an urgent need to increase dietary sources of this essential nutrient through mandatory food fortification practices.

Through the Canadian Food and Drug regulations, vitamin D fortification of fluid milk (35-45 IU/100 ml) and margarine (530 IU/100 g) is mandatory in Canada [153]. Other milk products including evaporated milk, powdered milk, goat’s milk and milks of plant origin (soy, rice and
other grains) require vitamin D and calcium fortification [131]. Vitamin D fortification is optional in the United States for fluid milk, breakfast cereals and fruit juices (40-140 IU/serving) [131]. Policy makers need to change the food regulatory system in Canada to increase food fortification practices and allow much higher levels of vitamin D fortification. There is a good range of evidence that vitamin D is bioavailable when added as a fortificant to orange juice [141, 142], bread [26, 143], cheese [29] and casein micelles [158]. Cheese is a good vehicle for providing vitamin D nutrition where previous studies have demonstrated that a cheese-like matrix is suitable for vitamin D₃ fortification [29]. Cheese is widely consumed around the world, its per capita consumption in Canada has increased by 44 percent between 1984 and 2003 [144]. According to the Canadian Food Trends to 2020, cheese consumption is expected to increase nearly 30 percent by 2020 [144]. However, milk consumption is expected to decline 15 percent by 2020 [144]. The forecasted increase in cheese sales are mainly due to the popularity of Italian, Mediterranean, and Mexican styles of cooking and from sales of cheese-containing foods such as pizza.

It is well known that cooking may result in some vitamin and mineral losses. Little is known about the effect of cooking on bioavailability of vitamin D from fortified mozzarella cheese. This is especially a concern with Mozzarella cheese which is commonly used in pizza. The heat of cooking can alter the matrix of Mozzarella cheese, and potential ingredients in pizza could sequester the vitamin D to prevent absorption in the gut.

In a double blind, randomized trial, our objective was to assess the effect of high temperature pizza baking on the bioavailability of vitamin D₃ fortified Mozzarella cheese. We tested the following hypotheses, set a priori: 1) vitamin D₃ is bioavailable from pizza baked with fortified Mozzarella cheese and heat does not significantly break down vitamin D₃; and 2) the change in serum 25(OH)D (from baseline to 10 wk) will be significantly greater in the high-dose vitamin D treated group (28000 IU/wk) compared to the low dose vitamin D-treated group (200 IU/wk).

5.2 Materials and Methods

The study was reviewed and approved by the Research Ethics Board of George Brown College (Toronto, Ontario, Canada), and of Mount Sinai Hospital (Toronto, Ontario, Canada). All the participants signed a written consent prior to enrolment in the study.
Subjects and Recruitment Criteria

Our study cohort was a convenience sample consisting of students and staff from George Brown College, the Centre for Hospitality & Culinary Arts in Toronto, Canada. The study was advertised at George Brown College through use of advertisements and classroom announcements to the students and staff. Participant eligibility was assessed through email contact for those who responded to our advertisements seeking healthy female and male subjects between 18 and 70 y of age for a vitamin D-pizza bioavailability study. Participants were excluded from the study if they reported any of the following criteria: 1) previous diagnosis of any medical conditions (kidney/liver damage, malabsorption conditions such as Crohn’s disease) that might affect vitamin D metabolism; 2) use of vitamin D supplementation in excess of 1000 IU/d; 3) travel to a sunny destination or use of tanning beds within the month prior to and during the study; 4) use of any medications (steroids, anticonvulsants, etc) that might interfere with vitamin D metabolism; 5) pregnancy; 6) unwillingness to commit for follow up. Between December 2011 and January 2012, we recruited 102 eligible healthy men and women at George Brown College of multi-racial ethnic backgrounds living in Toronto (latitude 43ºN).

Study Design

The 102 eligible subjects were randomly assigned to receive weekly servings of pizza meals baked with fortified Mozzarella cheese delivering either 200 IU or 28,000 IU vitamin D₃ per 28 g of cheese. (Figure 5.1) The pizza meals were consumed once a week for a total of 8 weeks at the Centre for Hospitality and Culinary Arts, George Brown College. Our weekly dose protocol was equivalent to 30 IU/d vitamin D₃ in the low dose group and 4000 IU/d vitamin D₃ in the high dose group [124].

The supplementation protocol began in the second week of February 2012 and ended in the fourth week of March 2012, a time of the year during which cutaneous synthesis of vitamin D from sun exposure is negligible in Toronto (latitude 43ºN) [168]. The subjects completed a total of 10 visits by the end of the study. The first and the last visits required venous blood and urine sample collections for biochemical measures which required the subjects to attend Mount Sinai Hospital (Toronto, Canada). Between visits 2 to 9, subjects attended the culinary studio at George Brown College to consume their weekly servings of pizza meals for a total of 8 visits.
Baseline and final serum 25(OH)D concentrations were measured to assess the bioavailability of vitamin D in pizza baked with low (200IU) or high (28,000IU) vitamin D₃ dose of Mozzarella cheeses. The primary outcome was the comparison of serum 25(OH)D concentrations between the low dose and high dose group at the 10 wk time point. Secondary outcome measurements included serum calcium, parathyroid hormone (PTH), creatinine, phosphate, alkaline phosphatase (ALP), human chorionic gonadotropin (HCG), as well as urine calcium, creatinine, and phosphate.

Subjects were randomly assigned to either the low or high dose vitamin D-treatment group using Microsoft Excel (2010) to create randomly permutated blocks of 6 allocations. Numerical codes were assigned to each subject and the corresponding cheese package such that both the
investigators and the subjects did not know whether their pizza meal was baked with the low or the high dose vitamin D₃ fortified Mozzarella cheese. The code was revealed to the researchers once recruitment, data collection and laboratory analyses were complete.

**Pizza Preparation Method/ Materials**

The two different doses of Mozzarella cheeses were industrially manufactured by Agropur Cooperative using standard cheese making methodologies. The fortified Mozzarella cheeses provided by Agropur Dairy Inc. were shipped to the Food Innovation and Research Studio (FIRS), George Brown College. The Mozzarella cheeses were shredded, portioned, packaged in sterile food bags, and stored in a freezer at -7 °C for later use throughout the study. The low and the high dose Mozzarella cheeses were handled in separate work areas to eliminate contamination and mix-up. All pre-portioned cheeses (28 g) were packaged into individual sterile food bags with blinding codes and the corresponding subject name. The vitamin D concentrations of the cheeses remained stable over the course of the study. The high and low dose Mozzarella cheese had identical colour and taste. Cook colour and flavour of pizza was not affected by vitamin D fortification.

Each pizza consisted of; (i) 120 g pizza dough; (ii) 25 g pizza sauce; (iii) 30 g pizza topping; and (iv) 28 g fortified Mozzarella cheese. The pizza dough was supplied by a bakery and delivered to the FIRS weekly, portioned into 120 g pieces, and frozen for later use. The pizza sauce was made in house in 10 litre batches and frozen in ½ litre portions for use throughout the study. The pizza topping was created at FIRS, and prepared fresh every pizza production day. The pizza flavour throughout the study was a tomato bruschetta. Every week on the day of the pizza protocol, the thawed and already portioned pizza dough was rolled out to a specific size such that 28 g of fortified cheese was sufficient without the addition of extra unfortified cheese. The dough was organized onto parchment paper on a baking tray. The pizza sauce and bruschetta topping were added to all pieces of dough on the baking tray. The subject’s blinding code was then written on the parchment paper beside the pizza dough. Their corresponding cheese, which was already portioned out, and labelled with blinding code number and the subject name, was then added on top of the bruschetta topping. Pizzas were baked at 375°F (190°C) for 8-10 minutes and removed from the oven. The pre-labelled paper plates with the subject names and blinding codes, were
laid out and the pizzas were placed on their respective plates. Compliance to dosing was monitored through returning the empty plates after the subjects had consumed their pizza meals.

**Measurements**

Anthropometric measurements (i.e., age, weight and height) were taken for each subject at the baseline visit. Serum 25(OH)D concentrations were measured by competitive chemiluminescence immunoassay (DiaSorin) on the automated LIAISON analyser (Stillwater, MN). The assay has a limit of detection of 3.75 nmol/L, an intra-assay coefficient of variation (CV) of 10%, and an inter-assay CV of 16%. To minimize assay variation, all samples from a single subject were analysed within the same run. Serum PTH, calcium, phosphate, creatinine, HCG, as well as urine calcium, phosphate, and creatinine were measured on the Modular Analytics Serum Work Area (Roche).

**Race/Ethnicity information**

Ethnicity was assessed through a self-reported questionnaire that asked subjects to specify a single race/ethnicity category; Native American/Inuit, South American, Black African, European, South Asian, East Asian, and West Asia/Middle East.

**Statistical Analyses**

All data were analysed using SPSS software (version 13.0). The results are reported as mean ± SD. Comparisons of baseline characteristics between groups were done by independent-sample 2-tailed t tests. Within group changes in serum and urine biochemical responses at baseline versus final were analysed with paired 2-tailed t tests. Mean serum and urine biochemical outcomes, as well as changes (Δ) in serum and urine biochemical variables between groups were analyzed with independent-sample 2-tailed t tests. Between racial/ethnic group differences in mean baseline serum 25(OH)D concentration, as well as 25(OH)D response to dosing were analysed with one-way ANOVA followed by post hoc testing, Tukey’s honestly significant difference. Associations between biochemical measures were examined by means of the Pearson correlation coefficient. The criterion for significance was set at P < 0.05.
5.3 Results

Characteristics of subjects

The flow chart shows the recruitment process of the participants [Figure 5.1]. Of the 102 subjects that initially enrolled in the study, 96 subjects completed the supplementation protocol (47 subjects in the low dose vitamin D-treated group, and 49 subjects in the high dose vitamin D-treated group). All of 96 subjects completed the entire protocol with 100% compliance. If a subject was unable to attend their weekly session, double dose of cheese (48 g) was administered on the pizza meal at their subsequent visit. The baseline demographics of the subjects randomly assigned to each group is shown in Table 5.1. There were no significant differences between the baseline characteristics of the subjects enrolled in the study.

Serum 25(OH)D

The mean serum 25(OH)D concentration at baseline in the total sample was 46.5 ± 23 nmol/L [range 15.6-123 nmol/L; (n=96)]. There was no significant difference in baseline serum 25(OH)D concentration between groups (P=0.31). In the total sample at baseline, 16 (17%) of subjects had serum 25(OH)D concentrations below 25 nmol/L, 60 (63%) had serum concentrations below 50 nmol/L, and 87 (91%) had serum concentrations below 75 nmol/L. In the low dose vitamin D treated group, serum 25(OH)D concentration increased from baseline 49 ± 24 nmol/L to 54 ± 23 nmol/L over 10 wk period (P=0.003) [Table 5.2]. In the high dose vitamin D treated group, serum 25(OH)D increased substantially by over 100% from baseline to 10 wk (44 ± 22 nmol/L to 117 ± 23 nmol/L; P<0.0001) [Table 5.2]. Change in serum 25(OH)D concentration was calculated as the difference between baseline and final serum 25(OH)D levels [Δ25(OH)D; nmol/L]. The Δ25(OH)D in the low dose group was 5.1 ± 11.1 nmol/L compared to 72.9 ± 21.9 nmol/L in the high dose group (P<0.0001) [Figure 5.2]. BMI was not correlated with baseline serum 25(OH)D concentrations (r= -0.18; P=0.08), but Δ25(OH)D was negatively correlated with BMI (r= -0.33, P=0.024) in the high dose vitamin D treated group only. Furthermore, 25(OH)D response to dosing stratified by ethnicity did not differ either in the low dose vitamin D treated group (P=0.21) or the high dose vitamin D treated group (P=0.63).
Table 5-1 Demographics and baseline characteristics of subjects consuming vitamin D$_3$-fortified Mozzarella cheese baked with pizza$^1$

<table>
<thead>
<tr>
<th></th>
<th>Low Dose</th>
<th>High Dose</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>47</td>
<td>49</td>
<td>96</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td>27.8 ± 10.2</td>
<td>26.9 ± 10.2</td>
<td>27.4 ± 10.2</td>
</tr>
<tr>
<td><strong>BMI, kg.m$^{-2}$</strong></td>
<td>25.6 ± 5.70</td>
<td>25.8 ± 5.60</td>
<td>25.7 ± 5.70</td>
</tr>
<tr>
<td><strong>Sex (n)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
<td>25</td>
<td>53</td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td><strong>Ethnicity [n(%)]</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native American/ Inuit</td>
<td>0 (0)</td>
<td>1 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>European</td>
<td>27 (57)</td>
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<td>49 (51)</td>
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<tr>
<td>East Asian</td>
<td>8 (17)</td>
<td>9 (18)</td>
<td>17 (18)</td>
</tr>
<tr>
<td>South Asian</td>
<td>6 (13)</td>
<td>9 (18)</td>
<td>15 (16)</td>
</tr>
<tr>
<td>Black African</td>
<td>3 (6)</td>
<td>5 (10)</td>
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<tr>
<td>South American</td>
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<td>Middle Eastern</td>
<td>1 (2)</td>
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<td>3 (3)</td>
</tr>
<tr>
<td><strong>Vitamin D supplement use (n)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>400 IU/d</td>
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<td>8</td>
</tr>
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<td>600 IU/d</td>
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</tr>
<tr>
<td>1000 IU/d</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><strong>Dropouts [n(%)]</strong></td>
<td>[4(8)]</td>
<td>[2(4)]</td>
<td>[6(6)]</td>
</tr>
<tr>
<td><strong>Compliance (%)</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Serum 25(OH)D nmol/L</strong></td>
<td>48.9 ± 24.4</td>
<td>44.2 ± 21.1</td>
<td>46.6 ± 22.7</td>
</tr>
<tr>
<td>&lt;75nmol/L, n (%)</td>
<td>42 (89)</td>
<td>45 (92)</td>
<td>87 (91)</td>
</tr>
<tr>
<td>&lt;50nmol/L, n (%)</td>
<td>26 (55)</td>
<td>34 (69)</td>
<td>60 (63)</td>
</tr>
<tr>
<td>&lt;25nmol/L, n (%)</td>
<td>6 (13)</td>
<td>10 (20)</td>
<td>16 (17)</td>
</tr>
<tr>
<td><strong>Serum Calcium, mmol/L</strong></td>
<td>2.35 ±0.11</td>
<td>2.35±0.08</td>
<td>2.35 ± 0.10</td>
</tr>
<tr>
<td><strong>Urine Calcium, mmol/L</strong></td>
<td>3.70 ± 2.20</td>
<td>3.50 ± 2.30</td>
<td>3.60 ± 2.30</td>
</tr>
<tr>
<td><strong>Serum Creatinine, µmol/L</strong></td>
<td>67.9 ± 16.3</td>
<td>74.7 ± 13.7</td>
<td>71.3 ± 15.0</td>
</tr>
<tr>
<td><strong>Urine Creatinine, mmol/L</strong></td>
<td>14.9 ± 8.70</td>
<td>13.3 ± 6.70</td>
<td>14.1 ± 7.70</td>
</tr>
<tr>
<td><strong>Urinary Calcium/Creatinine excretion, mmol:mmol$^2$</strong></td>
<td>0.30 ± 0.20</td>
<td>0.30 ± 0.20</td>
<td>0.30 ± 0.20</td>
</tr>
<tr>
<td><strong>Serum Phosphate, mmol/L</strong></td>
<td>1.20 ± 0.20</td>
<td>1.20 ± 0.20</td>
<td>1.20 ± 0.20</td>
</tr>
<tr>
<td><strong>Urine Phosphate, mmol/L</strong></td>
<td>20.8 ± 11.4</td>
<td>18.7 ± 10.0</td>
<td>19.8 ± 10.7</td>
</tr>
<tr>
<td><strong>Serum PTH, pmol/L</strong></td>
<td>4.40 ± 1.40</td>
<td>4.90 ± 2.20</td>
<td>4.60 ± 1.80</td>
</tr>
<tr>
<td><strong>Serum ALP, U/L</strong></td>
<td>62.9 ± 17.7</td>
<td>64.9 ± 14.1</td>
<td>63.9 ± 15.9</td>
</tr>
</tbody>
</table>

$^1$Values are means ± SD. Baseline values did not differ among groups, $P > 0.05$.

$^2$ Urinary calcium to creatinine ratio, expressed in millimolar concentrations.
Table 5-2 Biochemical responses in subjects consuming vitamin D₃-fortified Mozzarella cheese baked with pizza

<table>
<thead>
<tr>
<th></th>
<th>Low Dose Group (n=47)</th>
<th>High Dose Group (n=49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Change</td>
</tr>
<tr>
<td>25(OH)D, nmol/L</td>
<td>48.9 ± 24.4</td>
<td>5.1 ± 11.1*a,b</td>
</tr>
<tr>
<td>Serum Calcium, mmol/L</td>
<td>2.35 ± 0.11</td>
<td>-0.02 ± 0.01*a</td>
</tr>
<tr>
<td>Urine Calcium, mmol/L</td>
<td>3.74 ± 2.24</td>
<td>-0.05 ± 2.65*a</td>
</tr>
<tr>
<td>Serum Creatinine, µmol/L</td>
<td>67.9 ± 16.3</td>
<td>-1.72 ± 9.20*a</td>
</tr>
<tr>
<td>Urine Creatinine, mmol/L</td>
<td>14.9 ± 8.73</td>
<td>-1.18 ± 9.61*a</td>
</tr>
<tr>
<td>Urinary Calcium/Creatinine ratio, mmol:mmol</td>
<td>0.30 ± 0.20</td>
<td>-0.02 ± 0.16*a</td>
</tr>
<tr>
<td>Serum Phosphate, mmol/L</td>
<td>1.15 ± 0.17</td>
<td>0.00 ± 0.2*a</td>
</tr>
<tr>
<td>Urine Phosphate, mmol/L</td>
<td>20.8 ± 11.4</td>
<td>1.33 ± 16.2*a</td>
</tr>
<tr>
<td>Serum PTH, pmol/L</td>
<td>4.36 ± 1.38</td>
<td>0.15 ± 1.65*a</td>
</tr>
<tr>
<td>Serum ALP, U/L</td>
<td>62.9 ± 17.7</td>
<td>-0.87 ± 10.4*a</td>
</tr>
</tbody>
</table>

*Values are means ± SD. Significant changes within a group denoted by *; significant differences in the change of a measure between groups denoted by different letters a,b; P< 0.05

Baseline serum 25(OH)D concentrations showed considerable variation according to self-reported ethnicity. Figure 5.3 shows the baseline serum 25(OH)D concentrations according to different ethnicity. The mean serum 25(OH)D concentration was 56.3 ± 22 nmol/L for Europeans (n=49); 37.2 ± 17 nmol/L for East Asians (n=17); 30.4 ± 16 nmol/L for South Asians (n=15); 30.5 ± 9 nmol/L for Black Africans (n=8); 35.1 ± 6 nmol/L for South Americans (n=3); and 57.2 ± 21 nmol/L for Middle Easterns (n=3). Due to small sample size, baseline serum 25(OH)D levels for South Americans and Middle Eastern were combined into one group denoted by ethnicity code number 3 (Figure 3). Only one subject reported to be of Native American/ Inuit origin with baseline serum 25(OH)D concentration of 106 nmol/L (excluded from ethnicity analysis). ANOVA analysis for mean serum 25(OH)D concentrations showed significant differences between multi-ethnic categories (P<0.0001). Tukey’s HSD post hoc test revealed that the significant difference in mean baseline serum 25(OH)D concentrations between multi-ethnic groups was mainly driven by the higher serum 25(OH)D level in Europeans compared to Black Africans (P=0.008), East Asians (P=0.014), and South Asians (P<0.0001). No significant pairwise differences were found between East Asians and South Asians (P=0.75). Furthermore, 25(OH)D response to dosing stratified by ethnicity did not differ either in the low dose vitamin D treated group (P=0.21) or the high dose vitamin D treated group (P=0.63).
Figure 5-2 Changes in serum 25(OH)D concentrations in the low dose vitamin D treated group (LD) and in the high dose vitamin D treated group (HD) from baseline to 10wk. The whiskers show the highest and lowest values, and the top line indicates the 75th percentile, the middle line indicates the median, the bottom line indicates the 25th percentile, and the + represents the mean, n=47 (LD) and n=49 (HD). Means without a common letter differ, P < 0.05. The points above and below the whiskers represent outliers.
Figure 5-3 Baseline serum 25(OH)D concentrations according to ethnicity. Ethnicity codes represent; (1) European, (2) Black African, (3) Middle Eastern and South American, (4) South Asian, (5) East Asian. The whiskers show the highest and lowest values, the top line indicates the 75th percentile, the middle line indicates the median, and the bottom line indicates the 25th percentile. The points above the whiskers represent outliers.

By the end of the study, only 7 [15%; (n=47)] subjects in the low dose vitamin D treated group attained final serum 25(OH)D concentration >75 nmol/L compared to 6 (13%) subjects at baseline. Furthermore, of these 7 subjects in the low dose group who attained final serum concentrations above 75 nmol/L were of European ancestry, had baseline 25(OH)D levels >70 nmol/L, and 5 of these subjects were taking vitamin D supplements (range 400-1000 IU/d). In the high dose vitamin D treated group, 48 [98%; (n=49)] subjects attained final serum 25(OH)D
levels >75nmol/L compared to 4 (8%) subjects at baseline. Of these 4 subjects in the high dose group who had baseline serum concentration > 75nmol/L; 2 subjects were of European ancestry with no vitamin D supplement use; 1 subject was South Asian, taking 1000 IU/d vitamin D supplement; and 1 Alaskan American/Inuit origin with no vitamin D supplement use.

**Biochemical Responses**

The changes in biochemical responses between the low dose and high dose vitamin D treated groups from baseline to 10 wk are summarized in Table 5.2. Mean serum PTH levels significantly decreased by 13% at 10 wk time point in subjects in the high dose vitamin D treated group (P= 0.008). There was no significant change in serum PTH levels in the low dose vitamin D group by 10 wk (P=0.54). Furthermore, in the high dose vitamin D treated group, serum PTH levels decreased by 24% in subjects who had baseline serum 25(OH)D < 30nmol/L, 7% decrease for those with baseline serum between 30-50nmol/L, and 5% decrease for those with serum 25(OH)D > 50nmol/L (Figure 5.4). Serum creatinine, phosphate, ALP, as well as urinary creatinine, calcium/creatinine ratio, and phosphate did not differ either within or between groups by the end of the study (P > 0.05) (Table 5.2).

Serum calcium remained unchanged in the low dose group (P=0.25), but decreased in the high dose vitamin D treated group from baseline to 10 wk (P=0.035). The change in serum calcium did not significantly differ between groups by the end of the study (P=0.6), and serum calcium concentrations remained within the normal reference range in both groups (2.2-2.6 mmol/L). None of the subjects developed hypercalcemia (serum calcium > 2.75 mmol/L) or hypercalciuria [calcium:creatinine ratio (mmol:mmol) > 1]. None of the subjects in either groups developed any adverse events during the supplementation protocol.
Figure 5-4 Changes in mean serum PTH levels according to baseline 25(OH)D status in subjects in the high dose vitamin D treated group. On average, final serum PTH levels decreased by 24% for subjects who had baseline serum 25(OH)D < 30 nmol/L; 7% decrease for those 30-50nmol/L; and 5% decrease for those > 50 nmol/L.
5.4 Discussion

To our knowledge, this study represents the first formal attempt to test the bioavailability and suitability of vitamin D₃ fortified Mozzarella cheese for baking which is popular among Italian, Mediterranean, and Mexican styles of cooking. Our results show that vitamin D is bioavailable from pizza baked with fortified Mozzarella cheese. The change in 25(OH)D levels from baseline to 10 wk was significantly greater in subjects receiving the high dose vitamin D pizza meals compared with subjects in the low dose vitamin D treated group. Our results are in accordance with our previous research reported by Wagner et al. on 25(OH)D response from fortified Cheddar cheese delivering 28,000 IU vitamin D₃ per weekly serving [29]. Likewise, our findings support the results reported by Natri et al who showed that vitamin D₃ was bioavailable from fortified breads, as well as stable during the dough baking process [143]. Although we did not directly measure the concentration of vitamin D₃ after the pizza baking process, the increase in serum 25(OH)D levels in our subjects taken together with the heat stability of vitamin D₃ in cheese and bread-like matrixes data published by Wagner et al, and Natri et al [1, 143], strongly suggests that vitamin D₃ in Mozzarella cheese is stable during the high temperature pizza baking process (375 °F/190 °C for 8-10min). The heat stability of vitamin D₃ during the baking process is further supported by the comparable bioavailability results reported by Wagner et al [29]. Wagner et al reported a rise in serum 25(OH)D levels of ~67nmol/L in subjects consuming weekly servings of Cheddar cheese fortified at 28000 IU vitamin D₃/serving which is equivalent to our findings of ~73 nmol/L increase in serum 25(OH)D levels in the high dose vitamin D treated group. We conclude that high temperature baking process (375 °F/190 °C for 8-10min) does not significantly breakdown vitamin D₃.

There were no reported case of any adverse events in either group, further confirming previous findings of safety of vitamin D supplementation at doses ≥ 4000 IU/d [12, 29, 113, 114]. Serum and urine calcium concentrations remained within the normal reference range, with no case of hypercalcemia or hypercalciuria in either group. PTH concentrations decreased over 10 wk in subjects receiving the high dose pizza meals only. Other biochemical responses including serum and urine phosphate, serum creatinine, and serum ALP remained unchanged in both groups. Hence, vitamin D₃ did not improve markers of bone and mineral metabolism in our subjects, but the supplementation protocol was only 2 months and our study cohort was mainly healthy young
adults. This would be more appropriate to further investigate in an older population with a longer supplementation protocol.

We chose to fortify Mozzarella cheese at 2 different vitamin D$_3$ dosing levels. The amount of vitamin D$_3$ in the higher dose cheese fortified at 28000 IU/28 g is substantially higher than what would be added to foods. The higher dose was chosen because of its practicality for a clinical evaluation of bioavailability that is safe and effective [29, 113]. The lower dose cheese fortified at 200 IU/28 g is more likely to represent a conventional dose that would be suitable for Health Canada as a consumer product. Although the low dose cheese did produce a rise in serum 25(OH)D by ~5nmol/L from baseline to 10 wk, this should be interpreted with caution. It has been reported that an additional 1 µg/d vitamin D$_3$ intake translates into 0.7-1.0 nmol/L increase in serum 25(OH)D levels [114]. In our study, subjects consuming the high dose pizza meals had an increment of ~0.73 nmol/L/µg for a 4000IU/d dose. The equilibrium increment of 0.73 nmol/L/ 1 µg in the high dose vitamin D treated group is in accordance with previous studies of oral vitamin D supplementation [113, 114]. This is an independent confirmation of the general magnitude of 4000 IU/d dose effect we describe here. Contrary to the high dose group, an increment of ~6.7 nmol/L/µg was attained in the low dose group receiving 30 IU/d vitamin D$_3$ dose. Based on an equilibrium increment range of 0.7-1.0 nmol/L/µg, one should expect an increment of ~0.5 nmol/L increase in serum 25(OH)D in the low dose vitamin D-treated group consuming the 200 IU/wk (~30 IU/d) dose. Since we did not include a placebo control group in the study design we are unable to assess the real effect of the low dose cheese compared to a placebo dose. In addition, the habitual dietary intake of subjects was not assessed, and no advised restrictions on vitamin D intake from other dietary sources were given. Moreover, vitamin D supplement use in the low dose group was more frequent than in the high dose group (Table 5.1). Although subjects were blinded to the dose they were receiving, enrolling in the study protocol might have increased their awareness by undertaking preventative efforts like increased adherence to vitamin D supplementation. To clarify, vitamin D supplementation up to 1000 IU/d was allowed during enrollment if the subject was already taking a vitamin D supplement prior to study enrolment. Vitamin D supplementation was not permitted during enrollment if the subject had not taken a vitamin D supplement initially.
In our study sample which represented a multi-ethnic subpopulation in Toronto, vitamin D insufficiency was highly prevalent particularly among those with darker skin pigmentation. The lowest baseline vitamin D status were seen in Black Africans and South Asians having equivalent baseline serum 25(OH)D levels of 30 nmol/L, followed by East Asians with 37 nmol/L baseline serum 25(OH)D concentration. Our study is consistent with Gozdzik et al reporting significantly different vitamin D status of East Asians and South Asians compared to their European counterparts [105]. Furthermore, a number of small studies have reported serum 25(OH)D levels to be consistently lower in persons with darker skin pigmentation, and data from the National Health and Nutrition Examination survey (NHANES) suggest that serum 25(OH)D levels are highest in whites, lowest in non-Hispanic blacks, and intermediate in Hispanic groups [173]. Overall, there is considerable evidence that darker skin pigmentation is associated with a lower serum 25(OH)D concentration for a given amount of UVB exposure. The habitual dietary intake was not assessed in our study protocol, hence we are unable to further assess the dietary intake of multi-ethnic subgroups in relation to their vitamin D status. Nevertheless, our data is suggestive of an urgent need to increase the vitamin D status of high risk ethnic subgroups living in Canada. The new RDA requirements set by the IOM is estimated to cover 97.5% of the population with no additional recommendations required for subpopulations that live in northern latitudes, with darker skin pigmentation, and with heavy clothing due to cultural practices. However, whether meeting the new RDA requirements will meet the needs of sub-populations who are at greater risk of vitamin D insufficiency is still unclear, and warrants further investigation.

In conclusion, consumption of pizza delivering 28000 IU vitamin D₃ per week safely and effectively increased the vitamin D status of our subjects and significantly decreased mean PTH levels. Serum 25(OH)D concentration ≥ 75 nmol/L was attained in 98% of our subjects in the high dose vitamin D treated group. The bioavailability results of our study validate the approach of fortifying Mozzarella cheese with vitamin D₃ and its suitability for real life use such as baking pizza. The results of our study provides further evidence for the urgent need to improve vitamin D status in young adults living in Canada (Southern Ontario), particularly among ethnic subgroups with darker skin pigmentation. To acquire the 600IU/day (15µg/day) RDA by diet alone is difficult. Policy makers need to change the food regulatory system in Canada to increase fortified foods options and allow much higher levels of vitamin D fortification. Increasing the
range of fortified food options for Canadians is a good strategy to increase vitamin D intakes of Canadians toward the new RDA since the current food supply does not provide sufficient amounts of vitamin D either occurring naturally or through regulated fortification practices.
Chapter 6 : General Discussion
Vitamin D insufficiency remains a leading health concern in the North American populations. The newly established DRIs for vitamin D pose a challenge for Canadians given the limitations of insufficient amounts of vitamin D present in foods and non-existent sun exposure in the winter months, particularly in regions with higher latitude. There is a strong evidence indicating that current vitamin D intakes in adults are insufficient to meet the new recommendations for vitamin D [23-26]. Policy makers at Health Canada should be aware that the only source of vitamin D for the majority of the Canadians is dietary sources either through food fortification or supplementation in the winter months. Considering variety of foods are eligible for vitamin D fortification, few foods and beverages have been fortified with vitamin D except for milk and margarine in Canada. Cross-sectional studies suggest that current fortification practices are not sufficient in preventing vitamin D insufficiency in the winter in Canada [29]. The level of vitamin D added to fluid milk is not adequate to raise serum 25(OH)D concentrations and bring forth an increase in serum 25(OH)D concentrations [112]. Moreover, based on the IOM’s final report, additional recommendations were not reported for sub-populations including those that live in northern latitudes, those with darker skin pigmentation, and those with heavy clothing due to cultural practices. Yet, those in greatest need of increased dietary sources of vitamin D due to factors such as aging and darker skin pigmentation have the lowest intakes of vitamin D which further contributes to their low serum 25(OH)D concentrations. In particular, the aboriginal populations in Canada have drifted away from their traditional diets to more western diets, and darker skinned immigrants of Asian ancestry are reported to have significantly lower serum 25(OH) D concentrations than whites and consume significantly lower vitamin D from milk, ready-to-eat cereals and dietary supplements [131, 174]. Therefore, it becomes clear that the extension of vitamin D fortification to additional foods is appropriate and necessary.

The timing of this research project is opportune with the recently revised dietary guidelines for vitamin D in 2011. As shown from previous studies [1, 29], cheese is an excellent candidate for vitamin D fortification. The low lactose content in cheese is ideal for individuals with lactose intolerance which is more prevalent in ethnic sub-populations. Dairy Farmers of Canada previously supported our research group to develop a method for fortifying Cheddar cheese with vitamin D. Wagner et al demonstrated that cheddar cheese was suitable for vitamin D₃ fortification, and the vitamin D₃ was equally bioavailable from fortified Cheddar cheeses as vitamin D in a liquid supplement [29]. For reasons outlined in Chapter 2.15, we chose to fortify
Cheddar and Mozzarella cheeses with vitamin D₃ using milk caseins as the delivery vehicle instead of vitamin D emulsified with polysorbate 80, to refine the conditions that optimally deliver all casein-bound vitamin D into cheese.

In the food science component of my research, the low dose fortification level at 200 IU/serving aimed to produce fortification at applied levels that may conform to the amount of vitamin D per serving that will be permissible for fortification per serving of food, whereas the high dose fortification level at 28,000 IU/serving was mainly methodological because of its practicality for a clinical evaluation of bioavailability that is safe and effective [29, 113].

Prior to industrial manufacturing of Cheddar and Mozzarella cheeses, we tested the feasibility of the fortification protocol in a cheese model system and documented over 90% recovery of vitamin D₃ in replicated experiments (data not shown). We further confirmed the retention of vitamin D₃ in industrially relevant setting where we found 92% and 90% of the vitamin D₃ added to the fortified cheesemilks was recovered in the fortified Cheddar and Mozzarella cheeses, respectively, with the remaining vitamin D₃ being entrained into the whey. We also showed the uniform distribution of vitamin D₃ in the cheeses and that the fortification process did not substantially alter the yields or chemical compositions of the cheeses. These results indicate that the fortification protocol using caseins as the delivery vehicle for the industrial production of vitamin D₃-fortified Cheddar and Mozzarella cheeses is feasible. Even though the fortification protocol was feasible, our results showed that 8% of the vitamin D₃ was lost to whey during Cheddar and Mozzarella cheesemaking processes. This did not prove our hypothesis that binding of vitamin D₃ by casein proteins prevent loss of vitamin D₃ into whey. The partial loss of vitamin D₃ in the whey is suggestive of potential vitamin D₃ interactions with the fat globules, as well as β-lactoglobulin which is a major whey protein [156, 157]. However, the low amounts of vitamin D₃ present in the whey did suggest that vitamin D₃ remained significantly bound to caseins which resulted in near full recovery of vitamin D₃ in the cheeses. The 8% loss of vitamin D₃ in the Cheddar cheese whey was less than that of Wagner et al who reported 16% of the vitamin D₃ being entrained in the Cheddar cheese whey. This could potentially suggest that binding of vitamin D₃ to caseins resulted in lower loss of vitamin D₃ in whey. Furthermore, as a preliminary study, we measured the relative binding efficiency of vitamin D₃ by casein proteins. Using ultrafiltration technique to estimate the amount of “bound vitamin D₃” versus “unbound vitamin
we found that 85% of vitamin D₃ remained in casein suspension, and remaining 15% was detected in the supernatant (data not shown, analyses were duplicated). Although, the ultrafiltration technique is an indirect measure of index for vitamin D₃ binding to caseins, it could serve as an independent confirmation for the tight association between vitamin D₃ and caseins. However, it is methodologically difficult to measure the fraction of vitamin D₃ that remained bound to casein proteins or those dissolved with the milk fats during cheese making process.

The food science component of my research had some limitations. Firstly, it was not feasible for us to repeat the industrial manufacturing of fortified Cheddar and Mozzarella cheeses but we replicated experiments using the cheese model system prior to industrial cheese productions. Secondly, we did not fortify cheese using the conventional vitamin D emulsion (polysorbate 80) to be able to directly compare the efficiency of the two methods under the same experimental conditions. Thirdly, although we produced Cheddar and Mozzarella cheeses fortified at applied levels which is likely to conform to the amount of vitamin D per serving that will be permissible for fortification, our extraction method was incapable of detecting the lower concentrations of vitamin D₃ in Cheddar and Mozzarella cheeses fortified to 200 IU/serving. It is difficult to get an accurate sampling of such low amount of vitamin D, particularly in the whey sample with such large volume together with low vitamin D concentration, hence making it analytically challenging. However, we used the vitamin D₃ retentions obtained in the higher dose cheeses to estimate retention for the low dose cheeses, given that the cheesemaking process were identical under the same manufacturing conditions. Using the same fortification protocol, there is no reason to suspect that a lower fortification level would result in a significant change in vitamin D₃ retention. In addition, the absolute amount of vitamin D₃ added to unfortified cheesemilks was used to approximate the concentration of vitamin D₃ in the low dose cheeses. Fourthly, we did not test the stability of vitamin D₃ in the fortified Mozzarella cheese under heat treatments matched to the conditions during pizza baking process. However, from the previously obtained data, Wagner et al found no changes in vitamin D₃ content after heating full-fat and low fat Cheddar cheeses either at 100 °C for 12 minutes or 232 °C for 5 minutes. Lastly, we did not assess the stability of vitamin D₃ in the high dose fortified Cheddar cheese under short or long term storage. However, previous studies have reported no loss of vitamin D₃ over 9 months ripening period [1, 164].
With the current state of knowledge, it is difficult to identify whether vitamin D interacts with fat globules (given its fat soluble nature) or caseins during cheesemaking process. Since we were unable to fully protect the loss of vitamin D₃ in the whey, future studies could investigate the mechanism of vitamin D retention in cheese-curd matrix. The mechanism of vitamin D retention could be better elucidated with cheese made from reduced fat milk to investigate vitamin D retention in cheese void of fat matrix. Furthermore, future studies could assess whether vitamin D retention is affected by the porosity of the rennet gel and subsequent cheese curds which may be highly influenced by the cheesemaking process.

In the clinical nutrition part of my research, we showed that vitamin D₃ is bioavailable from fortified Mozzarella cheese baked with pizza. Consumption of pizza baked with the higher dose Mozzarella cheese fortified to 28000 IU/serving produced an increase in serum 25(OH)D that was significantly greater than the increase obtained with consumption of pizza baked with Mozzarella cheese fortified to 200 IU/serving. Lastly, the higher dose fortification of Mozzarella cheese was safe and effective because it did not adversely affect serum or urine calcium levels or other markers of bone and mineral metabolism, and resulted in a significant reduction in PTH levels, which is beneficial for bone. Together with the food science research data, the bioavailability results validate the approach of fortifying Mozzarella cheese with vitamin D₃ which could be readily incorporated into cooking.

In subgroup analyses, we also found that baseline serum 25(OH)D concentrations showed considerable variation according to self-reported ethnicity. Subjects with the highest baseline serum 25(OH)D levels were of European origin, and those with darker skin pigmentation (Blacks, Asians, and Middle Eastern etc) had lower baseline serum 25(OH)D levels. However, mean changes in serum 25(OH)D in response to dosing did not differ significantly between ethnic subgroups. The findings obtained in our study is consistent with that of Gozdzik et al reporting significantly different vitamin D status of East Asians and South Asians compared to their European counterparts [105].

There are also some limitations to the bioavailability study. Firstly, we did not directly measure the concentration of vitamin D₃ in cheeses following pizza baking process. But taking together the data showing the heat stability of vitamin D₃ in cheese and bread-like matrixes reported by
Wagner et al, and Natri et al [1, 143], we are confident in saying that vitamin D₃ in Mozzarella cheese was stable during the high temperature pizza baking process (375 °F/190 °C for 8-10min). The heat stability of vitamin D₃ during the baking process is further supported by the comparable bioavailability results obtained in our study to bioavailability results reported by Wagner et al [29]. Secondly, there was a discrepancy in the increment range for serum 25(OH)D levels between subjects in the high dose and low dose group. The lower dose cheese fortified at 200 IU/serving was chosen to represent a conventional dose that would be suitable for Health Canada as a consumer product. However, since we did not include a placebo control group in the study design, we are unable to assess the real effect of the low dose cheese compared to a placebo dose. Thirdly, we assessed bioavailability using change in serum 25(OH)D before and after the study. A more direct way to assess bioavailability would be to measure the absorption of vitamin D₃ itself. However, vitamin D₃ itself possesses a shorter circulating half-life with a wide distribution throughout body tissues compared to 25(OH)D, thereby making its measurement problematic [124]. Lastly, the habitual dietary intake of subjects was not assessed, and no advised restrictions on vitamin D intake from other dietary sources were given. This could have potentially effected the serum 25(OH)D levels, particularly in subjects in the low dose group. Future studies could potentially include a placebo group in a study design to test and validate the efficiency of low dose cheese fortified at 200 IU/serving in raising serum 25(OH)D concentrations compared to placebo treatment.

In summary, the fortification protocol using caseins as a delivery vehicle for vitamin D₃ did not result in optimization of the full retention of vitamin D₃ in cheese, but yielded near full recovery of over 90% retention of vitamin D₃ in Cheddar and Mozzarella cheeses. In addition, use of milk caseins is a more natural way to deliver vitamin D as a nutrient versus synthetic additives. Furthermore, vitamin D₃ is bioavailable from fortified Mozzarella cheese baked with pizza suggesting that the high temperature baking process (375 °F/190 °C for 8-10min) does not significantly breakdown vitamin D₃. Our findings could have important implications in increasing fortified food options for Canadians who are currently consuming insufficient amounts of vitamin D from dietary sources. In addition, rising immigration rates have created many changes in dietary intakes of Canadians. Particularly, milk is infrequently used by Asians and Middle Eastern subpopulation due to high prevalence of lactose intolerance, which may partly explain declining milk sales. Furthermore, analyses of the Canadian Health Measures
Survey data revealed that Canadians in other racial group (Non-white) consumed milk significantly less frequently than did those classified as white [23, 110, 111]. Current cheese sales are benefiting from the popularity of Mediterranean and Mexican styles of cooking, and from sales of cheese based such as pizza. With the rising immigration rates, growth in cheese sales will continue to be very strong. Mozzarella cheese does not contain vitamin D and is an excellent candidate for vitamin D fortification because it is widely consumed across different ethnic subpopulations, has a high nutritional value, can be consumed by most individuals with lactose intolerance, possesses a much longer shelf life than milk and could be readily incorporated into everyday meals. To our knowledge, this is the first research study to assess the fortification of Mozzarella cheese with vitamin D₃ and our data show that higher vitamin D intakes are efficient in raising vitamin D status across all populations, particularly those with ethnic backgrounds including East Asians, South Asians, Blacks, and Middle Eastern. When implementing new fortification practices, Health Canada should consider these ethnic subpopulations in Canada who infrequently consume milk and should give serious considerations to their dietary habits in order to successfully increase the dietary vitamin D intakes across a broader population. Fortification of Mozzarella cheese with vitamin D₃ implemented through either mandatory or voluntary fortification practices will expose the general population to increased levels of vitamin D₃ in foods. Increasing options and levels of vitamin D₃ fortification in foods will offer most individuals in the general population to easily acquire the 600 IU/d RDA through their diet. Our study help to further validate the approach of vitamin D₃ fortification of cheese from both a food science and clinical nutrition perspectives. Consequently, the results obtained in my research project could lead to the introduction of vitamin D₃-fortified Mozzarella cheese into the food market. Vitamin D₃ fortification of Mozzarella cheese is feasible and is a pleasant way to provide vitamin D at meals. The type of fortification (voluntary or mandatory) and level of fortification will be largely at the discretion of Health Canada and food fortification policies.
References


